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PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Kousuke Tani, *et al.*
Appln. No.: 10/506,536
Filed: September 3, 2004

Atty. Ref.: Q83408
Group: 1626
Examiner: Susannah Chung

For: 8-Azaprostaglandin derivative compounds and drugs containing the compounds as active ingredient

DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir/Madam:

I, Yoko Kajimoto, do declare and state that:

I graduated from the University of Kyoto, Faculty of Agriculture, having received a Master's Degree of Life Science in Graduate School of Biostudies in March, 2002.

I understand the Japanese and English languages.

In the present specification, the term "alcohol" on page 108, line 9 from the bottom is correctly "amine". The term "alcohol" on page 109, line 8 is correctly "amine". A marked-up version of these corrections is attached.

I declare that these errors were inadvertently caused in the preparation of the English translation of the originally filed Japanese PCT specification.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date : November 22, 2006

Name : Y. Kajimoto
Yoko Kajimoto

AMENDNENTS TO THE SPECIFICATION

Please replace the second full paragraph from the bottom on page 108 with the following amended paragraph:

(1) The method via an acyl halide may be carried out, for example, by reacting carboxylic acid with an acyl halide (e.g., oxalyl chloride or thionyl chloride *etc.*) in an organic solvent (e.g., chloroform, dichloromethane, diethyl ether or tetrahydrofuran) or without a solvent at -20°C to reflux temperature. And then the obtained acyl halide derivative may be reacted with amine~~alcohol~~ in an inert organic solvent (e.g., chloroform, dichloromethane, diethyl ether or tetrahydrofuran) in the presence of a base (e.g., pyridine, triethylamine, dimethylaniline, dimethylaminopyridine or diisopropylethylamine *etc.*) at 0 to 40°C. As an alternative, the obtained acyl halide derivative may be reacted in an organic solvent (e.g., dioxane, tetrahydrofuran) using an alkaline aqueous solution (e.g., sodium bicarbonate, sodium hydroxide) at 0 to 40°C.

Please replace the second full paragraph on page 109 with the following amended paragraph:

(3) The method using a condensing agent may be carried out, for example, by reacting carboxylic acid with amine~~alcohol~~ in an organic solvent (e.g., chloroform, dichloromethane, dimethylformamide, diethyl ether or tetrahydrofuran) or without a solvent, in the presence or absence of a base (e.g., pyridine, triethylamine, dimethylaniline or dimethylaminopyridine), using a condensing agent (e.g., 1,3-dicyclohexyl carbodiimide (DCC), 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC), 1,1'-carbodiimidazole (CDI), 2-chloro-1-methylpyridinium iodide, or 1-propanephosphonic acid cyclic anhydride (PPA)), in the presence or absence of 1-hydroxybenzotiazole (HOBt), at 0 to 40°C.

The bond angle or the conformation associated with the use of these divalent bioisosteres may be an important factor associated with retention of biological activity. Table 19 shows a comparison of the antiallergy activity and bond angles of several divalent bioisosteric substitutions which have been investigated for a series of 4-(diarylhydroxymethyl)-1-[3-(aryloxy)propyl]piperidines (**32**, Figure 23).[44]

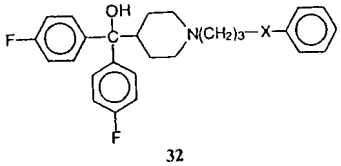

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Figure 23.

Table 19. Oral Antiallergy Activity in the Passive Foot Anaphylaxis Assay of Analogues Containing Varied Heteroatoms

compound	X	electronegativity[29]	bond angle[45] (deg)	passive foot anaphylaxis assay (10 mg/Kg)[a]
32a	−O−	3.51	108.0	+++
32b	−S−	2.32	112.0	+
32c	$-CH_2-$	2.27	111.5	+
32d	−NH−	2.61	111.0	+

[a] Aminophylline orally at 100 mg/kg was used as a positive control and assigned a biological response of (++); (−) not significantly different from negative control group at $p < 0.05$ as determined by the Dunnett's t test; (+) activity between positive and negative groups; (++) activity equivalent to positive control group; (+++) activity greater than positive control group.

These compounds were tested using the passive foot anaphylaxis assay which is an IgE-mediated model useful in the detection of compounds possessing antiallergic activity. A significant correlation between biological activity and electronegativity was observed for these analogues.

Another illustration of divalent bioisosteric linkers is observed in the study of inhibitors of the nuclear factor of activated T cells (NFAT)-mediated transcription of β-galactosidase.[46] T cells are essential components of the immune response. They are activated upon contact with foreign substances, or antigens, present on invading organisms. One of the earlier events that occurs after T cells recognize a foreign antigen is the induction of the interleukin-2 (IL-2) gene. IL-2 is an essential autocrine growth factor for T cells and its appearance marks the commitment of the T cell toward activation. These activated cells release a variety of bioactive molecules which initiate a cascade of events which initiate an immune/inflammatory response. The region 257−286 base pairs upstream of the IL-2 structural gene binds to a protein, the nuclear factor of activated T cells-1 (NFAT-1), prior to IL-2 gene transcription. NFAT-1 is expressed in relatively few cells besides T cells and is markedly upregulated upon stimulation of the T cell receptor. This makes it a highly specific target within activated T cells. When the cell is activated, the NFAT-1 protein binds to the DNA at its recognition site and induces the transcription of β-galactosidase. This study evaluated some of the bioisosteric analogues of quinazolinediones (**33**, Figure 24) as potential immunosuppressive agents by their ability to inhibit β-galactosidase expression as summarized in Table 20. Here again the similar bond angles and electronegativities (Tables 19 and 20) of the −NH− and $-CH_2-$ bioisosteric linkers result in analogues which retain activity. In this study, the use of an oxygen atom as a bioisosteric linker, which has a marginally smaller bond angle and much greater electronegativity, results in an analogue with increased potency.

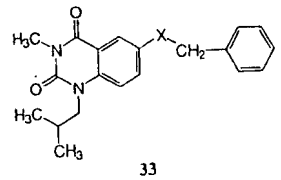

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Figure 24.

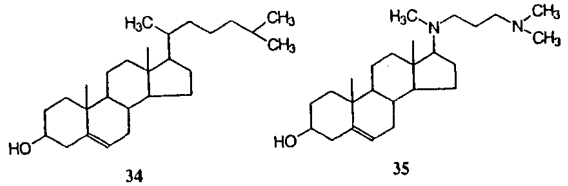
Table 20. Regulation of NFAT-1-Regulated β-Galactosidase Activity by Quinazolinediones

compound	X	IC_{50} (μM)
33a	−NH−	4.47
33b	$-CH_2-$	4.03
33c	−O−	2.5

Other divalent linkers that have been obtained as modifications of the above classical isosteres include higher oxidation states of the thioether linker resulting in sulfoxide and sulfone derivatives. These types of replacements will be discussed in the section on nonclassical isosteres.

C. Trivalent Atoms or Groups

A classical trivalent bioisosteric replacement is −CH= with −N=. This replacement has been widely used in the drug discovery process and has been further discussed among the ring equivalent class of classical bioisosteres. This replacement when applied to cholesterol (**34**) resulted in 20,25-diazacholesterol (**35**, Figure 25) which is a potent inhibitor of cholesterol biosynthesis.[47] The greater electronegativity of the nitrogen atom could be responsible for the biological activity of this bioisostere.



34 **35**

Figure 25.

Another trivalent substitution based on Erlenmeyer's definition of the similarity in the number of peripheral electrons would be the replacement of −P= (bond angle C−P−C = 100 ± 4°) with −As= (bond angle C−As−C = 96 ± 5°).[48] Arsenic is a classical bioisostere of nitrogen (Table 3). Arsenicals have received considerable attention due to their therapeutic significance. The oxidation of arseno compounds to arsenoxides is important in the bioactivation of a number of chemotherapeutic arsenicals. One of the first drugs used clinically was arsphenamine (**36**, Figure 26). The activity of arsphenamine against the syphilis organism was attributed to its oxidized metabolite oxophenarsine. However,

Reference 1

Comparison of Analgesic Effects of Isosteric Variations of Salicylic Acid and Aspirin (Acetylsalicylic Acid)

LEON THOMPKINS * and K. H. LEE *

Abstract □ A reliable and sensitive method was used to compare the analgesic activities of salicylic acid and aspirin (acetylsalicylic acid) and several phenoxy substituted isosteric pairs. Those isosteric compounds studied did not show analgesic activity. The analgesic activity of aspirin was more than twofold greater than that of salicylic acid.

Keyphrases □ Aspirin—analgesic effects of isosteric variations, compared to salicylic acid isosteres □ Salicylic acid—analgesic effects of isosteric variations, compared to aspirin isosteres □ Analgesics—comparison of effects of isosteric variations of salicylic acid and aspirin □ Salicylates—comparison of analgesic effects of isosteric variations of salicylic acid and aspirin

Of the three major pharmacological actions of salicylic acid and aspirin (acetylsalicylic acid), their quantitative difference is most apparent in their analgesic effect. This difference in potency in experimental animals and in humans has been observed by several workers (1–5) and has been primarily responsible for the suggestion that aspirin's activity is not due to conversion to salicylic acid (6–8).

Although this suggestion was first made more than 50 years ago, there have been no attempts to resolve the question of whether aspirin is analgesic in its own right by using comparative analgesic studies of isosteric structural modifications. This seems especially significant in that isosteric substitution of $-CH_2-$ or —NH— for the phenoxy oxygen in aspirin would result in compounds stable or less susceptible to hydrolysis, which has been a major problem in assessing the relative effects of aspirin and salicylic acid on the biological system. Such studies might provide indications of the structural requirements for analgesic activity and information concerning the mechanism by which the salicylates exert their pharmacological effects.

Perhaps one reason that such studies were not carried out was the lack of reliable bioassay procedures for testing nonnarcotic analgesics. Aspirin and salicylic acid show activity as antinociceptive agents in many tests (9). However, most of them require high doses of salicylate to produce a minimal effect, are not clearly definitive, or are unreliable.

Guzman *et al.* (3) introduced a method that employs intraarterial injections of bradykinin in dogs, producing vocalization and other autonomic and behavioral reactions, to assess the activity of nonnarcotic and narcotic analgesics. In animals that respond, this method is superior to other analgesic tests if one considers overall reliability, sensitivity, and definitiveness. This method was later adapted to rats, and similar results were found (10). However, while Guzman *et al.* (3) used primarily the vocalization as the index to the pain response after the bradykinin injections in dogs, the latter workers described struggling, dextrorotation of the head, and occasional vocalization as indexes.

The effects of intraarterial injections of bradykinin into rats were used in this study to assess the analgesic activities of several phenoxy-substituted isosteric pairs of salicylic and acetylsalicylic acids. The analgesic effects of the following pairs were studied: acetylsalicylic acid (I) and salicylic acid (II), *N*-acetylanthranilic acid (III) and anthranilic acid (IV), acetylthiosalicylic acid (V) and thiosalicylic acid (VI), and 2-acetonylbenzoic acid (VII) and 2-methylbenzoic acid (VIII).

EXPERIMENTAL

Reagents—The following were used: thiosalicylic acid[1] (mp 163–165°), *N*-acetylanthranilic acid[1] (mp 184.5–187°), acetylsalicylic acid[2] (ACS reagent, spectrophotometrically pure), homophthalic acid anhydride[3] (mp 139–141°), salicylic acid[3], chloroform[4] (technical), anthranilic acid[5], pyridine[6] (reagent grade), 2-methylbenzoic (*o*-toluic) acid[7], and sterile sodium chloride injection[8] USP (0.0009 g/ml). All other chemicals were reagent grade.

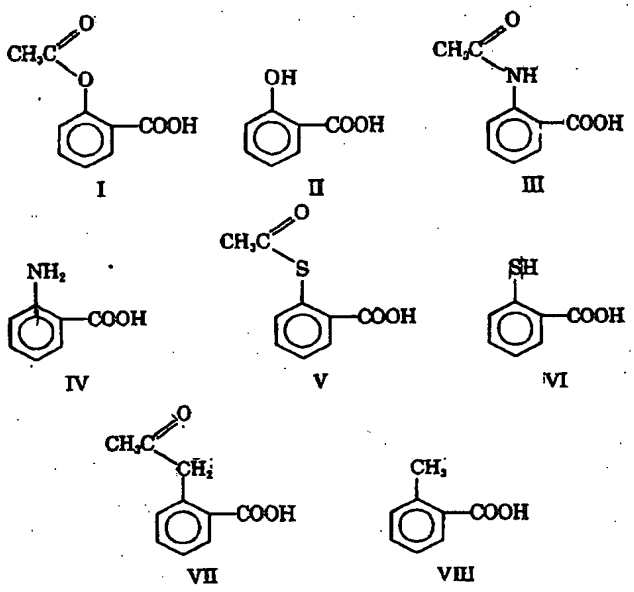
Purification Procedures—Thiosalicylic acid was recrystallized from ethanol and water as described in the literature (11). Pyridine was dried, distilled, and stored over sodium hydroxide pellets (12). Chloroform was purified, dried, and distilled (13). The distillate was used immediately.

Synthesis of Acetylthiosalicylic Acid—This compound was prepared using the method of Hinsberg (14). The crude product was dried over phosphorus pentoxide at room temperature under vacuum. The dried product was recrystallized several times from benzene until needles melting at 125° were obtained.

Synthesis of 2-Acetonylbenzoic Acid—This compound was prepared using the methods described by Schnekenburger (15, 16). These methods involve acetylation of homophthalic acid anhydride to obtain the 4-acetylhomophthalic acid anhydride, followed by alkaline hydrolysis with simultaneous decarboxylation of the product to 2-acetonylbenzoic acid (15). Colorless needles with the melting point of 2-acetonylbenzoic acid (122°), as described by Schnekenburger (16), were obtained.

Restraining Cage—For the measurement of analgesic activity, a restraining cage was designed in which the reactions of the animal to intraarterial injections of bradykinin could be observed and intraperitoneal injections of the test drug could be made (Fig. 1). The overall length of this cage was 20.3 cm (8 in.), and the distance between vertical supports was 7.6 cm (3 in.). The openings in the

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] City Chemical Corp., New York, N.Y.
[3] Baker analyzed reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] Baker grade, J. T. Baker Chemical Co., Phillipsburg, N.J.
[6] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.
[7] Eastman grade, Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.
[8] Provided by the University of California Hospital Pharmacy, San Francisco, Calif.



front and rear supports were holes with a depth of 3.7 cm (1.47 in.) and a width of 4.4 cm (1.75 in.). The top was removable; once the animal was positioned, the top was secured with wingnuts to hold the rat firmly but comfortably (Fig. 2). This cage was found to be suitable for these experiments.

Measurement of Analgesic Activity—Male Sprague–Dawley or Wistar rats were used in all experiments. A modification of the procedure of Deffenu *et al.* (10) was used in the testing of the analgesic activity of the various isosteric pairs. The rats, 230–320 g, were anesthetized with ether in an open mask and the right common carotid artery was catheterized, using a catheter consisting of two fused lengths of heparinized polyethylene tubing with inside diameters of 0.58 (PE 50) and 0.28 (PE 10) mm.

The catheter was inserted centripetally, passing through the subcutaneous tissues so that the portion of the tubing with the smaller diameter was inserted into the artery while that with the larger diameter protruded from the top of the head. After the tubing was anchored and the incision was closed, the tubing was opened to check blood flow, filled with heparin (40 units/ml in sterile saline injection USP), shortened so that the rat could not reach it, and plugged with a metal plug. At least 24 hr was allowed for each rat to recover prior to testing.

Rats were placed in the cages, and polyethylene 50 tubing extensions were attached to the projecting catheter *via* a hollow metal tube prepared from a length of No. 23 hypodermic needle. The tubing was taped to the top of the cage so that the animal would be unaware of the experimenter's handling of the distant end. Then 0.2 ml of normal saline was injected very slowly from the carotid artery, and no reaction of the animal was observed. This was followed by an injection of 0.5 μg of bradykinin in 0.2 ml of normal saline to determine if the animal was responsive to bradykinin. In about 99% of the rats, vigorous struggling and dextrorotation of the head were observed. Rats that were unresponsive to this dose of bradykinin were not used further. The sodium salt of the drug to be tested was injected intraperitoneally in normal saline. The volume of the injection was 0.25 ml/100 g or the smallest volume that could completely dissolve the salt.

Ten minutes after the injection of the drug, the animals were challenged with another 0.5-μg dose of bradykinin, preceded by a normal saline injection, and the animals' responses were carefully watched. The same procedure was repeated at 10-min intervals until the bradykinin effect reappeared.

If no effect was seen with a dose level of 1000 mg/kg for a particular drug, intraarterial studies with a dose level of 100 mg/kg were made. The intraarterial injections of the drugs were made in normal saline (0.5 ml/rat) *via* the same catheter used for the bradykinin injection. Otherwise, the procedure was the same as for the intraperitoneal injections.

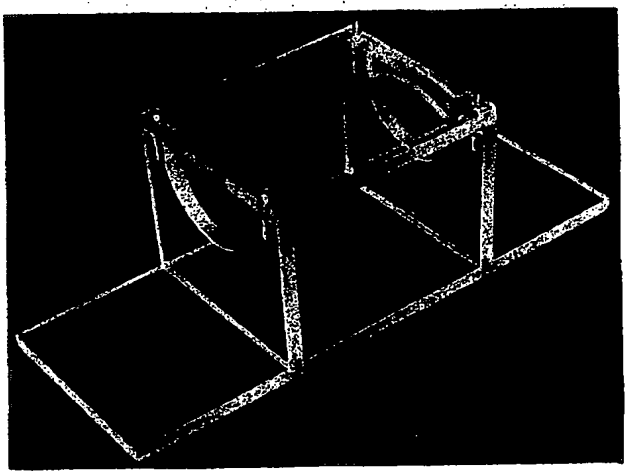


Figure 1—*Rat cage.*

The criterion chosen for evaluating protection was the disappearance of the bradykinin effect after two consecutive doses of bradykinin. The doses of drugs that showed no protection after five consecutive doses of bradykinin were assumed to be nonanalgesic in these studies. Each rat received one drug at one dose level, and five rats were used for each dose level. If a drug was found to be toxic, the LD_{50} was determined.

The ED_{50} and LD_{50} values were obtained using Thompson's method (17) of interpolation between moving averages.

RESULTS AND DISCUSSION

A summary of the data obtained in these experiments is contained in Table I, including the responses of the groups of rats to various doses of the drugs tested. Salicylic and acetylsalicylic acids showed clearcut analgesic activity *versus* bradykinin injections, intraperitoneally. Acetylsalicylic acid showed analgesic activity at a much lower dose level when intraarterial injections of the drug were made.

It is difficult to interpret the results obtained with *N*-acetylanthranilic and anthranilic acids. Both of the drugs seemed to afford protection *versus* bradykinin injections at lower dose levels intraperitoneally. However, at higher intraperitoneal dose levels and at a dose level of 100 mg/kg intraarterially, no analgesic activity was demonstrated. No other analgesic drugs demonstrating such anomalous behavior have been described. It is assumed that these two compounds have no analgesic activity as indicated in Table II, where the ED_{50} values for compounds showing analgesic activity and the LD_{50} values for compounds found to be toxic are reported.

Both Tables I and II show the toxicity of acetylthiosalicylic and thiosalicylic acids, the nonacetylated compound being more toxic. This toxicity was characterized by severe tremors and hyperventilation followed by a violent struggle just before death. All animals thus affected died within 50 min, and *rigor mortis* set in rapidly.

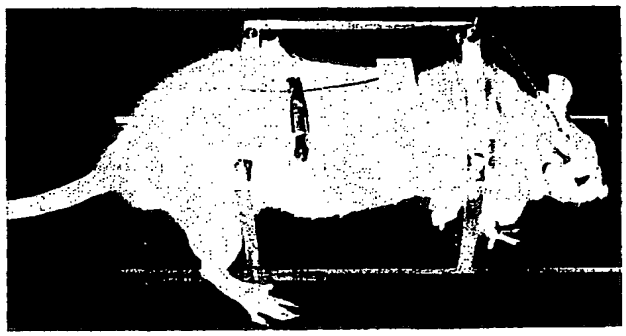


Figure 2—*Rat cage with rat.*

Table I—Effects of the Injection of Various Salicylate *Isosteres versus* Intraarterial Injections of Bradykinin in Rats

Drug	Number of Rats Tested	Dose of Drug, mg/kg	Route[a]	Number Protected *versus* Bradykinin	Number of Deaths
Acetylsalicylic acid	5	31.6	ip	1	0
	5	46.4	ip	1	0
	5	68.1	ip	2	0
	5	100.0	ip	5	0
	5	10.0	ia	4	0
Salicylic acid	5	68.1	ip	1	0
	5	100.0	ip	2	0
	5	147.0	ip	2	0
	5	215.0	ip	5	0
N-Acetylanthranilic acid[b]	5	100.0	ip	2	0
	5	215.0	ip	1	0
	5	464.0	ip	2	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0
Anthranilic acid[b]	5	100.0	ip	0	0
	5	215.0	ip	2	0
	5	464.0	ip	0	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0
Acetylthiosalicylic acid	5	147.0	ip	0	0
	5	215.0	ip	0	3
	5	316.0	ip	0	4
	5	464.0	ip	0	5
Thiosalicylic acid	5	100.0	ip	0	0
	5	147.0	ip	0	3
	5	215.0	ip	0	4
	5	316.0	ip	0	5
2-Acetonylbenzoic acid	5	100.0	ip	0	0
	5	464.0	ip	0	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0
2-Methylbenzoic	5	100.0	ip	0	0
	5	464.0	ip	0	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0

[a] ip = intraperitoneal, and ia = intraarterial. [b] See discussion.

In contrast to other methods, the bradykinin injection causes no injury and shows that the receptors for pain are chemosensitive (18). The sensitivity and reliability of this method are indicated by the fact that one could show the difference in potency of acetylsalicylic acid and salicylic acid (Table II). The procedure of injecting normal saline prior to injecting bradykinin allows one to compare the effects of nonstimulating and stimulating agents and, therefore, increases the sensitivity of the test.

The concept of isosterism has undergone several changes since its introduction in 1919. The term "isosteres" was first applied by Langmuir (19) to designate two molecules or ions having identical number and arrangement of electrons. Hinsberg (20) recognized even earlier that certain groups such as sulfur, viylene, and trivalent carbon and nitrogen could be interchanged in the aromatic ring without appreciably altering physical and chemical properties. Hinsberg's designation of such groups was "ring equivalents." Hückel (21) included other types of inorganic and organic groups under the equivalent designation, equating methyl, methylene, and methyne to —F, —N—, and —O—, respectively.

Grimm (22, 23) applied the term "pseudoatom" to groups such as —NH_2 and —OH since, in his opinion, addition of a proton (H^+) to the oxide ion results in the addition of a proton to the outer shell where it reaches equilibrium at or in the outer shell, and the result is thus a pseudo-F^- or the hydroxide ion. However, unlike the ring equivalents of Hinsberg, chemical properties of compounds obtained using the "hydride displacement law" might appear wholly different but may be similar in some physical properties.

Erlenmeyer (24) included both Grimm's concept of pseudoatoms and all elements of a particular group of the periodic table in his concept of isosteres, redefining the isosteres as "atoms, ions, or molecules in which the peripheral layers of electrons can be considered identical." Erlenmeyer was also one of the first to apply the isosteric principle to biological problems (25, 26).

Table II—LD_{50} and ED_{50} Values for Acetylsalicylic and Salicylic Acid Isosteres *versus* Intraarterial Injections of Bradykinin in Rats

Compound	Intraperitoneal LD_{50}, mg/kg	Intraperitoneal ED_{50}, mg/kg	Intraarterial, ED_{50}, mg/kg	95% Confidence Limits
Acetylsalicylic acid	—	64.9	—	47.9–88.00
Salicylic acid	—	138	—	84.1–226
N-Acetylanthranilic acid	—	1000	100	—
Anthranilic acid	—	1000	100	—
Acetylthiosalicylic acid	224	—	—	176–285
Thiosalicylic acid	153	—	—	120–194
2-Acetonylbenzoic acid	—	1000	100	—
2-Methylbenzoic acid	—	1000	100	—

Since the development of more sophisticated methods of treating atoms and molecules (*e.g.*, quantum mechanics), it is recognized that the concept of isosterism disregards some important factors such as size, shape, polarity, and hybridization, and the concept is of little theoretical importance to the pure chemist because of the unpredictability of its application. However, it has great value for the medicinal chemist, since biological properties of isosteres are more similar than their physical or chemical properties.

Since isosteres often have similar biological properties, Friedman (27) introduced the term bioisosterism to apply to compounds "which fit the broadest definition of isosteres and have the same type of biological activity." He included under this term isosteres with antagonistic activities, since these compounds may be acting by a similar mechanism. The broadest definition of the term isostere would, of course, include the ring equivalents of Hinsberg, the isosteres of Langmuir, the equivalents of Hückel, and the pseudoatoms of Grimm, all of which have been found to have utility in the search for more desirable, biologically active compounds. Thus, Erlenmeyer found that the isosteres *p*-aminodiphenylamine, *p*-aminodiphenylmethane, and *p*-aminodiphenyl ether all show similar antigen activity. Halogen isosteres of the antihistamine, tripelennamine, show greater antihistaminic activity when F and Cl are substituted for one aromatic hydrogen in the parent compound (28). Several other examples of the utility of the concept of isosterism in medicinal chemistry can be found in the literature (27, 29).

It is unusual that none of the isosteres was effective in producing analgesia, especially since most of the groups are closely related in size. Apparently, the receptors are very sensitive to the electronic and steric configurations of acetylsalicylic and salicylic acids. However, it has been demonstrated again that the analgesic activity of acetylsalicylic acid is more than twofold greater than that of salicylic acid.

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Fluorocarbon Aerosol Propellants IV: Pharmacokinetics of Trichloromonofluoromethane following Single and Multiple Dosing in Dogs

SARFARAZ NIAZI and WIN L. CHIOU [x]

Abstract □ An intravenous dosage form of trichloromonofluoromethane, a common aerosol propellant, was formulated in polyethylene glycol 400 for single and multiple dosing to unanesthetized dogs. A three-compartment open model was proposed for disposition of this compound in dogs with average half-lives of 3.2, 16, and 93 min for three disposition phases. This finding is contrary to several reports where blood levels were monitored for shorter periods. A computer analysis of tissue compartment distribution following a single dose showed that about 2 hr was required to achieve pseudodistribution equilibration, following which more than 90% of the dose remaining in the body was retained in tissue compartments. Pulmonary clearance and volumes of distribution were calculated considering first-pass effect through the lung. The volume of distribution was approximately six times the body weight in terms of blood concentrations, and about 30% of the propellant was cleared from blood passing through the lung in each cycle. Disposition of propellant followed dose-independent kinetics after multiple dosing, and accumulation in tissues continued for a much longer period, resulting in high tissue compartment levels.

Keyphrases □ Trichloromonofluoromethane—pharmacokinetics following single and multiple dosing, dogs □ Fluorocarbon aerosol propellants—pharmacokinetics of trichloromonofluoromethane following single and multiple dosing, dogs □ Aerosols—pharmacokinetics of trichloromonofluoromethane following single and multiple dosing, dogs □ Propellants—pharmacokinetics of trichloromonofluoromethane following single and multiple dosing, dogs □ Pharmacokinetics—trichloromonofluoromethane, single and multiple doses, dogs

The volatile fluorocarbons have been widely used as aerosol propellants in commercial aerosol packages in this country. Although these compounds have been generally considered nontoxic and inert, several studies (1–3) have claimed that a variable degree of cardiac damage can be caused following their inhalation. An often used argument in favor of low toxicity of fluorocarbons is that these compounds are not absorbed to any significant extent when inhaled from a commercial aerosol product and that the small fraction absorbed is eliminated very fast from the body, decreasing the possibility of any toxic reaction (4–6). Unfortunately, the conclusions drawn from most previous studies monitoring blood levels of fluorocarbons following their inhalation from commercial aerosol products were not based on sound pharmacokinetic principles and do not reflect the true disposition pattern of these compounds as was recently discussed (7).

The objective of the study reported here was to demonstrate that, contrary to the established belief, trichloromonofluoromethane, one of the most commonly used fluorocarbon propellants, has a longer biological half-life than previously thought and under-

Reference 2

Example 1: Suppressive Activity of the Compound Used in the Present Invention on Uterine Contraction Induced by $PGF_{2\alpha}$, PGE_2, Vasopressin, Oxytocin and Methacholine in Nonpregnant Rats

(Pharmacological Experiment)

Nonpregnant female rats in which sexual cycle was synchronized by subcutaneous administration of estradiol (500 μg/kg) on the day before the date of the experiment were used. Uterine horn was incised and an open-end catheter filled with a physiological saline solution was inserted thereinto. Intrauterine pressure was recorded through a pressure transducer and a strain pressure amplifier. After stabilization of the intrauterine pressure, an inducer (100 μg/kg of $PGF_{2\alpha}$, 100 μg/kg of PGE_2, 10 U/kg of vasopressin, 100 mU/kg of oxytocin or 50 μg/kg of methacholine) was intravenously administered so as to raise the intrauterine pressure. After the rise in the intrauterine pressure by the inducer was confirmed once again, a test substance, i.e. (5Z,9ß,11α,13E)-17,17-propano-11,16-dihydroxy-9-chloro-20-norprosta-5,13-dienoic acid lysine salt (EP2 agonist; hereinafter, abbreviated as the compound A), was continuously and intravenously administered in a dose of 1, 3 or 10 μg/kg/min. After 30 minutes from the initiation of the continuous administration of the test substance, an inducer was administered. Suppressive activity by the test substance was evaluated in view of a suppressive rate to the state before administration of the test substance by determining the area under the intrauterine pressure curve which was raised by administration of the inducer (the area under the curve after

5 minutes from the administration in the case of $PGF_{2\alpha}$ and PGE_2; that after 2 minutes from the administration in the case of vasopressin; that after 10 minutes from the administration in the case of oxytocin; and that after 1 minute from the administration in the case of methacholine).

(Results)

Suppressive rates of the compound A to the uterine contraction induced by $PGF_{2\alpha}$, PGE_2, vasopressin, oxytocin and methacholine in the nonpregnant rats are shown in the following table.

Table 1

Inducing Condition for Uterine Contraction		Compound A		
Inducer	Dose	1 μg/kg/min	3 μg/kg/min	10 μg/kg/min
$PGF_{2\alpha}$	100 μg/kg	37.8%	68.7%	85.4%
PGE_2	100 μg/kg	N. D.	N. D.	51.0%
Vasopressin	10 U/kg	N. D.	N. D.	46.7%
Oxytocin	100 mU/kg	N. D.	N. D.	67.3%
Methacholine	50 μg/kg	N. D.	N. D.	62.1%

N. D. = no data

(Consideration)

In nonpregnant rats, the compound A which is an EP2 agonist suppressed the $PGF_{2\alpha}$-induced uterine contraction in a dose-dependent manner. Besides $PGF_{2\alpha}$, the compound A also suppressed the uterine contraction induced by PGE_2, vasopressin, oxytocin or methacholine. Therefore, the possibilities that an EP2 agonist is effective for dysmenorrhea caused by

contraction of uterine muscle and that it is able to be expected to have higher effect than NSAID which suppresses the production of prostaglandin and antagonists such as oxytocin and vasopressin were suggested.

Reference 3

総　説

眼圧下降薬としてのプロスタグランジン研究の現況

松　尾　俊　彦
岡山大学医学部眼科学教室

要　約

人や種種の動物にプロスタグランジン（prostaglandin D_2, E_2, F_2alpha）を点眼すると，眼圧下降がみられる．その機序はuveoscleral outflowの促進による．人眼の毛様体筋には，プロスタグランジン受容体が存在し，その主体はEP 2受容体である．プロスタグランジンは，この毛様体筋にある受容体に作用し，毛様体筋を弛緩させることにより，uveoscleral outflowを増加させると考えられる．眼圧下降に関与するプロスタグランジン受容体を明らかにすることにより，炎症などの副作用のより少なく，眼圧下降効果のより大きなプロスタグランジン製剤の開発が期待される．（日眼会誌　97：289—296，1993）

キーワード：プロスタグランジン，受容体，眼底下降

A Review

Prostaglandins as Ocular Hypotensive Drugs

Toshihiko Matsuo
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Abstract

Prostaglandins can lower intraocular pressure when they are applied topically to the eye. This pressure-lowering effect is mediated by enhancement of uveoscleral outflow. Human ciliary muscle has binding sites (receptors) for prostaglandins and the EP2 receptor subtype is the predominant prostanoid receptor found in this muscle. Prostaglandins are thought to bind to these receptors located on the ciliary muscle, induce its relaxation, and promote the uveoscleral outflow. It is important to know the receptor type involved in the pressure reduction to design a new drug with less adverse effect and more potency. (J Jpn Ophthalmol Soc 97 : 289—296, 1993)

Key words: Prostaglandins, Receptor, Intraocular hypotension

I　はじめに

プロスタグランジンを点眼すると，まず一過性の眼圧上昇を起こし，引き続いて長時間持続する眼圧下降を来す．初期にみられる一過性の眼圧上昇は，プロスタグランジンの種類，使用する動物の種，あるいは投与するプロスタグランジンの量により，起こったり起こらなかったりする．また，一過性の眼圧上昇にともなって前房内炎症がみられる．人に点眼した場合では結膜に対する刺激症状がある．

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現在までに，prostaglandin F_2alpha の眼圧下降作用は，人，猿，犬，猫，兎で確認され[1)~8)]，prostaglandin E_2の作用は，猿，猫で[9)~11)]，また，prostaglandin D_2の作用は，人，猫，兎で確認されている[11)~15)]．プロスタグランジン研究の難点は，その作用（たとえば炎症を起こすか起こさないか）が，動物種により著しく異なり[16)]，動物実験の結果をそのまま，人にあてはめることができない点である．炎症を惹起することが少なく，人に使える眼圧下降薬として，prostaglandin F_2alpha および prostaglandin D_2系の化合物が有力である．

prostaglandin F_2alpha isopropyl ester は，prostaglandin F_2alpha をエステル化し，その脂溶性を高めた製剤である．脂溶性を高めたことにより，角膜に対する透過性が増し，前房に達しやすくなった．したがって，少量の点眼でも眼圧下降を起こし，炎症などの副作用をおさえることができるようになった．

炎症を来すことなく眼圧下降を起こす点眼薬として，この prostaglandin F_2alpha isopropyl ester が注目されている．この点眼薬の眼圧下降作用は，緑内障患者においても確認されている[17)18)]．また，これとは別の化合物 UF-021 が，日本国内で開発され[19)]，現在臨床治験が行われている．

II プロスタグランジン受容体

プロスタグランジンの眼圧作用機序を知るうえで，また，より副作用が少なく効果のある眼圧下降薬を開発するうえで，プロスタグランジン受容体の種類およびその分布を明らかにすることは大切である．また，プロスタグランジンの作用は，動物種間でかなり異なることより[16)]，プロスタグランジン受容体の人眼における分布を知ることは不可欠である．

プロスタグランジンの受容体（レセプター）は，生体内に存在するプロスタグランジンおよびその合成誘導体（agonist）に対する反応の違いに基づいて，以下のように薬理学的に大きく5つに分類されている[20)21)]．

EP 受容体：prostaglandin E_2に対して主に反応

FP 受容体：prostaglandin F_2alpha に対して主に反応

DP 受容体：prostaglandin D_2に対して主に反応

TP 受容体：thromboxane A_2に対して主に反応

IP 受容体：prostacyclin（prostaglandin I_2）に対して主に反応

EP 受容体は更に，EP 1，EP 2，EP 3 受容体の3種類に分類されている[22)23)]．生体内に存在するプロスタグランジンは，親和性の差こそあれ，1種類以上の受容体と反応する場合もある．たとえば，prostaglandin F_2alpha は，FP 受容体にも，EP 受容体にも結合しているようである．現在，各受容体に対してより特異的に反応する agonist が開発されつつあり，今後，プロスタグランジン受容体の薬理学的研究がすすむであろう．

プロスタグランジン受容体は，細胞膜に存在する蛋白質である[24)]．その受容体蛋白を生化学的に精製した報告がいくつかある．近年，thromboxane A_2受容体蛋白の遺伝子が，人血小板の cDNA library により分離され，その塩基配列が決定された[25)]．その結果，thromboxane A_2受容体は，G protein（GTP binding protein）に連結する受容体グループ G protein coupled receptor family[26)27)]に属することが明らかになった．つまりこのことは，プロスタグランジン受容体は，細胞内伝達系として，まず GTP 水解系と連結していることを示している．この G protein coupled receptor family に属する他の蛋白としては，視細胞のロドプシン，アドレナリン受容体などがある．

thromboxane A_2受容体蛋白の遺伝子を，人工的にアフリカツメガエルの卵細胞で発現させたところ，この受容体は thromboxane A_2には反応するが，他のプロスタグランジン（prostaglandin D_2，F_2alpha）には反応しなかった[25)]．このことは，さきほど述べた薬理学的分類のように，それぞれのプロスタグランジンに対して別個の受容体が存在することを示唆している．

III 人眼におけるプロスタグランジン受容体の分布

プロスタグランジン受容体がどこにあるかを知る方法の1つとして，放射性同位元素で標識したプロスタグランジンを用い，組織切片上で反応させ，オートラジオグラフィーを行い，プロスタグランジンが組織のどの細胞に結合したかをみる方法がある．対照としては，反応液に標識したプロスタグランジンとともに，その1,000倍から10,000倍の濃度を有する未標識のプロスタグランジンを加えて，標識プロスタグランジンの結合が阻害されることを確認する．また，未標識物質として，いろいろな結合能を持ったプロスタグランジン誘導体を用いると，結合部位（受容体）の性質を詳細に知ることができる．オートラジオグラフィーで得られたフィルム上の濃淡を画像解析により判定

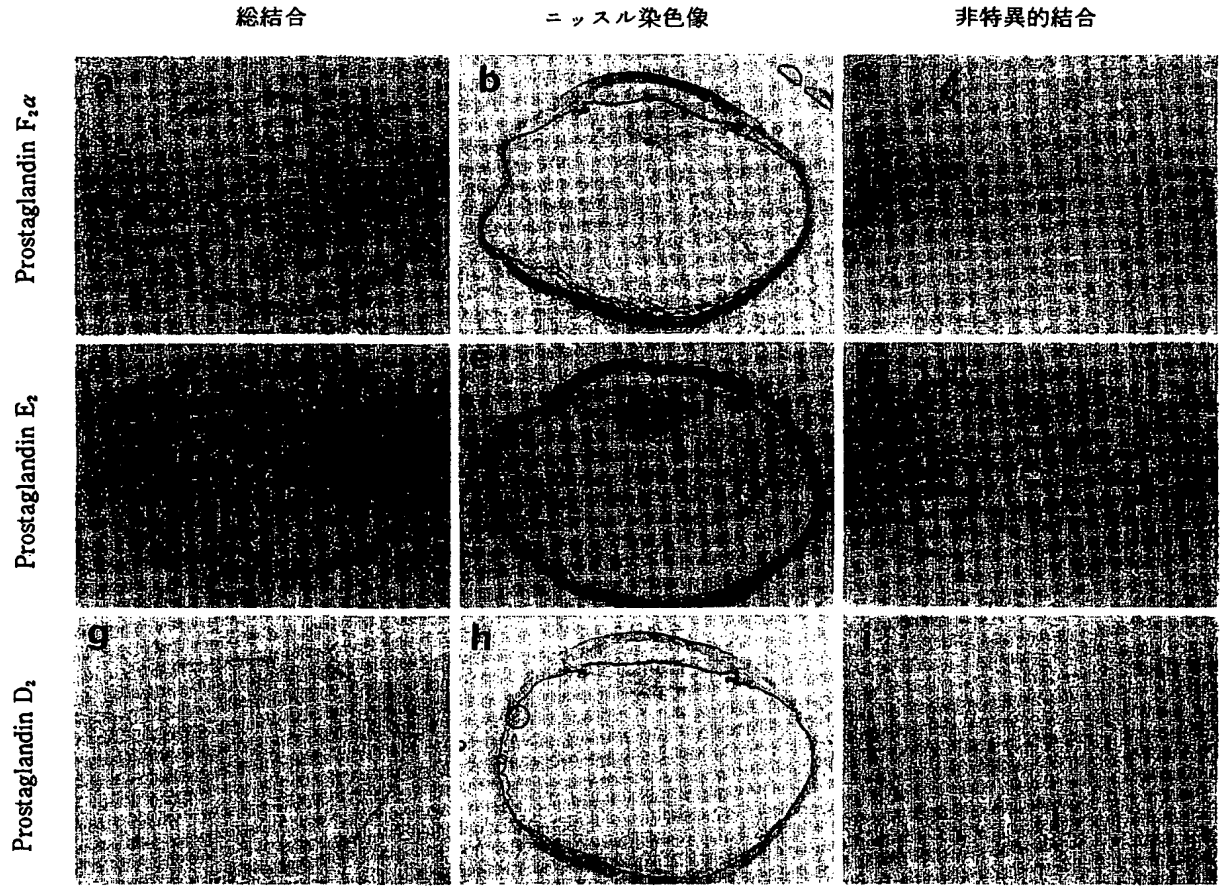

図1　人眼切片における［^{3}H］prostaglandin F_2alpha，E_2，D_2の結合部位．
毛様体筋，瞳孔括約筋，および網膜に結合部位がある．（文献28）29)から引用）
(a) 2 nM[^{3}H]prostaglandin F_2alpha，(b) 切片(a)のニッスル染色像，(c) 2 nM[^{3}H]prostaglandin F_2alpha＋10 μM prostaglandin F_2alpha，(d) 2 nM［^{3}H］prostaglandin E_2，(e) 切片（d）のニッスル染色像，(f) 2 nM［^{3}H］prostaglandin E_2＋10 μM prostaglandin E_2，(g) 2 nM［^{3}H］prostaglandin D_2，(h) 切片（g）のニッスル染色像，(i) 2 nM［^{3}H］prostaglandin D_2＋10 μM prostaglandin D_2

し，数値化することにより，薬物の結合曲線を描くことができるようになり，薬物の解離定数や，最大結合部位数を求めることができる．

人眼の凍結切片を用いて，上述した方法でオートラジオグラフィーを行うと，prostaglandin D_2，E_2，F_2alpha の受容体は毛様体筋，瞳孔括約筋に高濃度で存在し，その他，網膜にも存在することがわかった[28)29)]（図1）．この受容体は，毛様体筋のなかでも，longitudinal muscle に特に高濃度で存在していた．標識したプロスタグランジンの濃度を変え，得られたオートラジオグラフィーのフィルム上の濃淡を画像解析により数値化し，毛様体筋における各プロスタグランジンの結合曲線をえがいた．その結果，prostaglandin E_2に対する結合部位数（受容体数）が，prostaglandin F_2alpha および D_2に対する結合部位数に比べて多いことがわかった[29)]（図2）．

未標識物質として，各受容体に対してより特異的なagonist を用いて，どの程度各プロスタグランジンの結合が阻害されるかをみたところ，prostaglandin E_2 および F_2alpha の結合はともに，prostaglandin E_2および11-deoxy prostaglandin E_1（EP2 receptor agonist）により，もっとも効果的に阻害された[29)]（図3および図4）．一方，prostaglandin D_2の結合は，prostaglandin D_2によりもっとも強く阻害された[29)]（図5）．つまり，人眼毛様体にあるプロスタグランジン受容体は，EP2受容体が主体であり，小数ながら

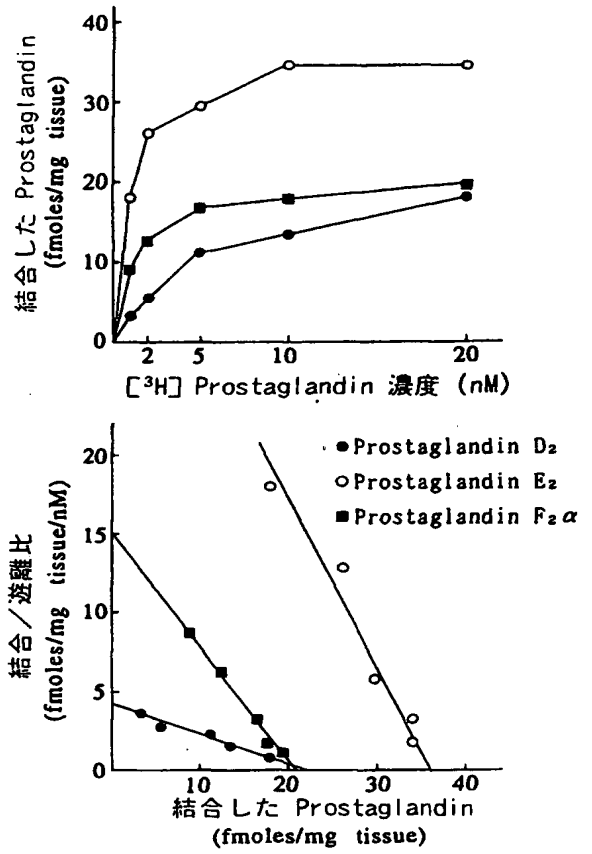

図2 人眼毛様体筋における [³H] prostaglandin F_2 alpha, E_2, D_2の結合曲線.
毛様体筋には, prostaglandin E_2に対する結合部位がもっとも多い. (文献29)から引用)

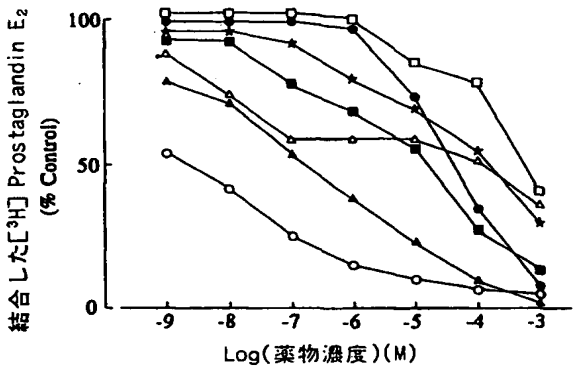

図3 人眼毛様体筋における [³H] prostaglandin E_2 結合に対する各種薬物による阻害効果.
[³H] prostaglandin E_2の結合は, prostagalandin E_2および11-deoxy prostaglandin E_1によりもっとも強く阻害される. (文献29)から引用)
● Prostaglandin D_2 ○ Prostaglandin E_2
■ Prostaglandin $F_2\alpha$ □ U46619
▲ 11-deoxy Prostaglandin E_1 △ Sulprostone
★ 17-phenyltrinor Prostaglandin $F_2\alpha$

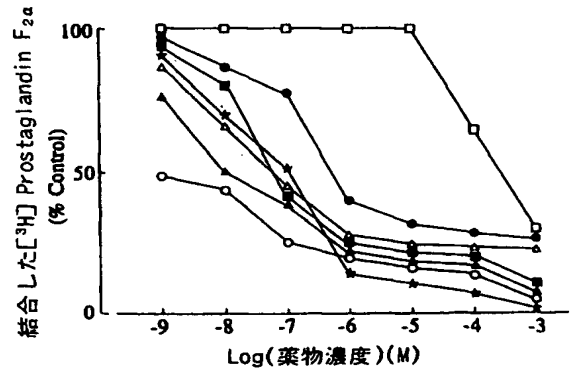

図4 人眼毛様体筋における [³H] prostaglandin F_2 alpha 結合に対する各種薬物による阻害効果.
[³H] prostaglandin F_2alpha の結合は, [3 H] prostaglandin E_2の結合同様, prostaglandin E_2および11-deoxy prostaglandin E_1によりもっとも強く阻害される. (文献29)から引用)
● Prostaglandin D_2 ○ Prostaglandin E_2
■ Prostaglandin $F_2\alpha$ □ U46619
▲ 11-deoxy Prostaglandin E_1 △ Sulprostone
★ 17-phenyltrinor Prostaglandin $F_2\alpha$

DP受容体も存在することを示している. このうち, どちらの受容体が, 眼圧下降に対してより重要な働きをしているかは, 現在のところ不明である. また, prostaglandin F_2alpha は, 主にEP 2受容体に結合していることになる.

なお, 猿および猫の眼球を用いて同様に調べたところ, prostaglandin F_2alpha に対する結合部位は, 人眼と同じく主に毛様体筋に存在することがわかった (図6).

IV プロスタグランジンの作用部位

猿を用いた実験によると, プロスタグランジンにより, 毛様体による房水産生量は変わらず[30)31)], また隅角線維柱体よりの房水排出量もかわらない[32)]. そして, プロスタグランジンによる眼圧下降作用は, ピロカルピンにより打ち消されるので, uveoscleral outflow を促進すると考えられている[33)–35)]. プロスタグランジン受容体は毛様体筋に存在することにより, この考えはさらに支持される.

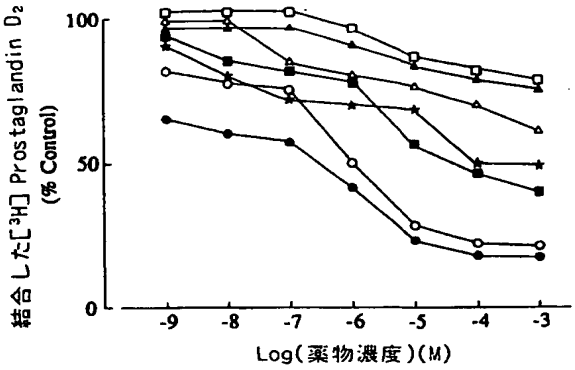

図5　人眼毛様体筋における [³H] prostaglandin D_2 結合に対する各種薬物による阻害効果.
[³H]prostaglandin D_2の結合は，prostaglandin D_2 によりもっとも強く阻害される．(文献29)から引用)

● Prostaglandin D_2　　○ Prostaglandin E_2
■ Prostaglandin $F_2\alpha$　　□ U46619
▲ 11-deoxy Prostaglandin E_1　　△ Sulprostone
★ 17-phenyltrinor Prostaglandin $F_2\alpha$

同じく猿を用いた実験により，プロスタグランジンを点眼すると，毛様体筋の線維の幅が狭くなり，その間隙が広くなることがわかっている[36]．また，プロスタグランジンの点眼により，毛様体筋線維間のコラーゲン線維が減少するという報告もある[37]．

一方，兎の毛様体筋を分離し，あらかじめ *in vitro* でカルバコールによって収縮させておき，これにプロスタグランジンを投与すると，毛様体筋が弛緩することが報告されている[38]．

以上のように，プロスタグランジンは毛様体筋に直接作用し，それを弛緩させることによって uveoscleral outflow を増加させるという証拠が集まりつつある．

猿の実験で，プロスタグランジンの点眼により眼圧は下がるが，調節緊張の程度には有意な変化はないことがわかっている[39]．人における点眼でも，特に調節の緊張度に変化はみられない[8][17]．毛様体筋は解剖学的には，その筋線維の走行により，longitudinal muscle, radial muscle, circular muscle の3つに分類される．そして longitudinal muscle が，主に uveoscleral

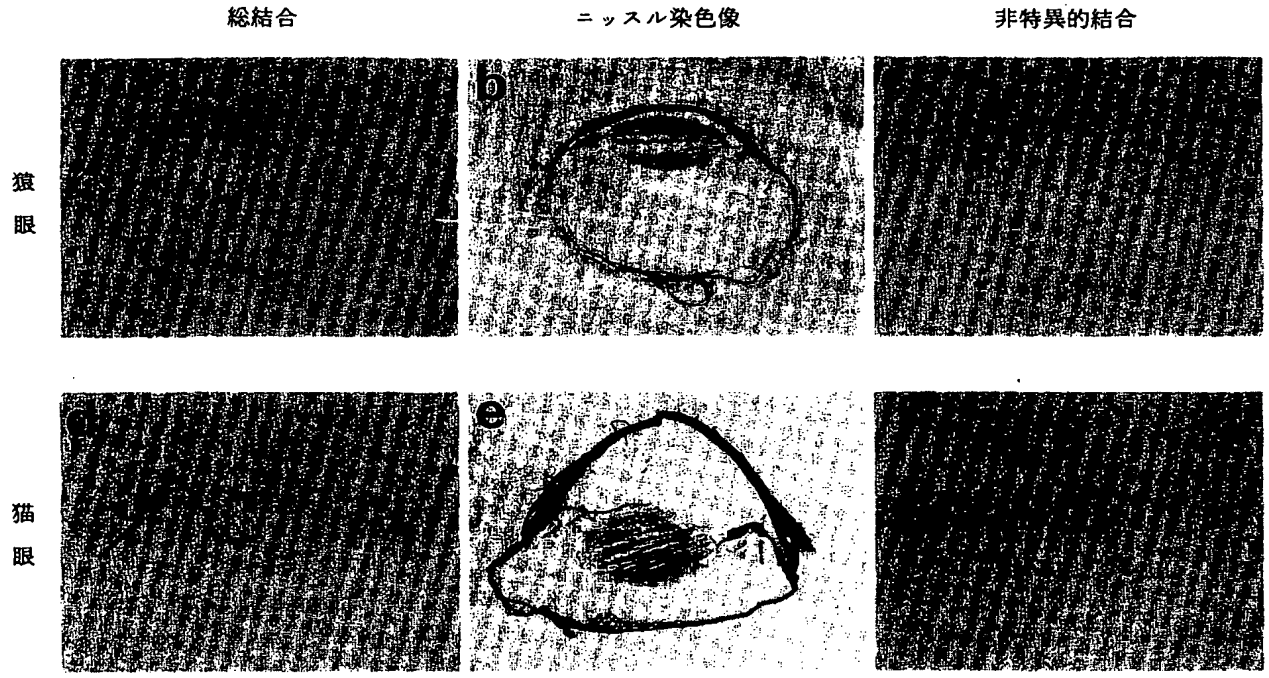

図6　猿眼 (a, b, c) および猫眼 (d, e, f) 切片における [³H] prostaglandin F_2alpha の結合部位.
人眼同様，毛様体筋に結合部位がある．
(a) 猿眼，10 nM [³H] prostaglandin F_2alpha，(b) 猿眼，(a)のニッスル染色像，(c) 猿眼，10 nM [³H] prostaglandin F_2alpha＋10 μM prostaglandin F_2alpha，(d) 猫眼，10 nM [³H] prostaglandin F_2alpha，(e) 猫眼，(d)のニッスル染色像，(f) 猫眼，10 nM[³H]prostaglandin F_2alpha＋10 μM prostaglandin F_2alpha

outflowの制御に関与し，circular muscleが，調節の制御の方により強く関与していると考えられている．プロスタグランジン受容体がlongitudinal muscleのほうに高濃度に存在することは，上述したように，プロスタグランジンが主にuveoscleral outflowの制御に関与しているとの考えに合致する．

V プロスタグランジンの生理的な役割

prostaglandin E_2, F_2alphaは，培養した人眼線維柱体内皮細胞により合成され，分泌されていることがわかっている[40][41]．このように，眼内でプロスタグランジンが合成され，それがどのような生理的な役割を果たしているのかについて以下に推察を述べる．

1つの考えとして，線維柱体の内皮細胞により合成され，房水中に分泌されたプロスタグランジンは毛様体筋に達し，そこで毛様体筋にある受容体に作用し，毛様体筋を弛緩させる．つまり，主な房水流出路にあたる線維柱体の内皮細胞が，別の房水流出経路uveoscleral outflowを調節している可能性がある．

一方，毛様体筋自身がプロスタグランジンを分泌し，自分自身に作用するautocline的な働きをしている可能性もある．毛様体上皮細胞がプロスタグランジンを産生している可能性もある[42]．

VI プロスタグランジン点眼薬の今後の展望

以上述べたように，プロスタグランジンは今までの眼圧下降薬とは，全く違った作用機序をもった薬物である．現在までにtimololの点眼と，prostaglandin F_2 alpha isopropyl esterの点眼との併用により，更なる眼圧下降効果が得られることがわかっている[43][44]．今後，これら従来の薬物では，眼圧が十分下がりきらない難治性の緑内障に対して，併用点眼薬として使える可能性がある．また，プロスタグランジンの点眼による眼圧下降作用の持続時間が長いことより（人眼組織は，プロスタグランジン分解酵素をもたず[45]，プロスタグランジンは眼外に輸送系で排泄される[46][47]），その点眼回数が少なくてすむ可能性がある．従来の薬物の点眼回数がうまく守れない人に対して，処方できるかもしれない．

プロスタグランジン点眼薬のもっとも大きな問題点は，前房内炎症を起こしたり結膜刺激を起こしたりする炎症惹起作用である．眼圧下降に関与するプロスタグランジン受容体と，炎症反応に関与する受容体とは，その種類が異なっていると考えられる．そこで理論的には，炎症反応に関与する受容体には作用せず，眼圧下降に関与する受容体のみに作用する薬物をさがせばよいということになる．眼圧下降に関与するプロスタグランジン受容体の種類が明らかになり，それに基づいて，より副作用の少なく，より効果の大きいプロスタグランジン製剤を開発することが今後の課題である．

プロスタグランジン研究の機会をあたえてくださったカナダ国ブリティッシュ・コロンビア大学眼科学教室のMax S. Cynader教授および岡山大学医学部眼科学教室の松尾信彦教授に感謝します．

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Reference 4

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Molecular Characterization and Ocular Hypotensive Properties of the Prostanoid EP_2 Receptor

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ABSTRACT

The cloning of the genes that encode for prostaglandin (PG) receptors has resolved much of the complexity and controversy in this area by confirming the classification proposed by Coleman, et al. Two issues that remained unresolved were (1) the inability of the EP_2 agonist butaprost to interact with the cloned putative EP_2 receptor and (2) molecular biological confirmation of a fourth PGE_2-sensitive receptor, which was pharmacologically designated EP_4. In order to provide clarification, we attempted to clone further PGE_2-sensitive receptors. By using a cDNA probe that encodes for the human EP_{3A} receptor, a cDNA clone that encoded for a novel PGE_2-sensitive receptor was obtained by screening a human placenta library. This cDNA clone was transfected into COS-7 cells for pharmacological studies. The cDNA clone obtained from human placenta had only about 30% amino acid identity with cDNAs for other PG receptors, including those that encode for the previously proposed murine and human EP_2 receptors. Radioligand binding studies on the novel EP receptor expressed in COS-7 cells revealed that selective EP_2 agonists such as butaprost, AH 13205, AY 23626 and 19(R)-OH PGE_2 all competed with ^3H-PGE_2 for its binding sites, whereas selective agonists for other PG receptor subtypes had minimal or no effect. This receptor was coupled to adenylate cyclase and EP_2 agonists caused dose-related increases in cAMP. It appears that the cDNA described herein encodes for the pharmacologically defined EP_2 receptor. Ocular studies revealed that AH 13205 decreased intraocular pressure in normal and ocular hypertensive monkeys by a mechanism that does not appear to involve inhibition of aqueous humor secretion.

INTRODUCTION

The structural identity of the biologically active constituents of human seminal fluid and irin were originally described approximately three decades ago as E and F series prostaglandin [1,2]. It was quickly discovered that prostaglandins were potent, locally acting hormones with a remarkably broad spectrum of activity. Despite their clear biological significance, little progress in understanding the pharmacological basis of PG activities was made until quite recently. The topic of prostanoid receptors for a long time encompassed strikingly different speculations, some of which excluded contemporary receptor concepts and contemplated interaction with the plasma membrane lipid bilayer [3]. More conventional prostanoid receptor postulates were based on physiological responses [4], second messenger coupling [5], and the functional potency rank-order of natural PGs and their synthetic analogs [4,6]. The latter classification proposed distinct receptors for each of the major prostanoids, based on a characteristic potency rank order in those tissues which appeared to contain a homogeneous population of prostanoid receptors. Receptors that preferentially interacted

with prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin I_2 (PGI_2) and the endoperoxide prostaglandin H_2 (PGH_2) and thromboxane A2 (TxA_2) were designated DP, EP, FP, IP and TP, respectively. Since its conception in 1982 [6], this classification was extended to include subdivision of the EP receptor [4]. More importantly, during the past three years this classification has been supported almost in its entirety by studies describing the cloning and expression of cDNAs that encode the prostanoid receptors [7-13]. Molecular cloning has established prostanoid receptors as members of the G-protein coupled receptor superfamily. Molecular biological studies have also revealed complexities which were not appreciable from previous biochemical and pharmacological studies on prostanoid receptors. Alternative mRNA splicing variants for EP_3 [14-15] and TP [16] receptors have been reported which, by virtue of altering amino acid sequences in the carboxyl terminus, can be an important determinant of G-protein interaction.

One unresolved issue that emerged as a result of cDNA cloning was the identity of an adenylate cyclase coupled PGE_2-sensitive receptor. This was described as the EP_2 receptor, despite the inability of the selective EP_2 receptor agonist butaprost to compete with PGE_2 for the expressed receptor sites. For the murine receptor this was ascribed to a possible species difference [17], whereas the likely existence of a butaprost-sensitive receptor was postulated based on the reported pharmacological activity of this PGE analog in human tissues [10]. We describe herein the molecular characterization of a butaprost-sensitive EP receptor subtype which is coupled to adenylate cyclase. The identification of a PGE_2-sensitive receptor with characteristics consistent with the pharmacologically defined EP_2 will also advance knowledge regarding the physiological significance of PGs in the eye. This is a complex area that requires much further study.

One intriguing aspect of PG ocular physiology is the ability of so many receptor selective prostanoid analogs to lower intraocular pressure in non-human primates, which may be regarded as surprising. The possibility that this reflected the presence of several different prostanoid receptors in the eye, at anatomical locations where they could mediate an ocular hypotensive response, was supported by radioligand binding studies in tissues that were similarly responsive to multiple PG analogs [18]. The recent cloning of cDNAs that encode for these receptors supports the postulate that multiple prostanoid receptor subtypes may be involved in regulating intraocular pressure. Evidence that EP_2 stimulation results in ocular hypotension in primates is provided by examining the effects of the selective EP_2 agonist AH 13205 [19].

MATERIALS AND METHODS

The cDNA for the human EP_2 receptor was obtained by screening a human placenta cDNA library (Clontech) by plaque hybridization using a cDNA probe containing the complete coding sequence for the human EP_{3A} receptor [15] and labeled with ^{32}P. The placenta library, constructed in λgt11, was plated at a density of approximately 25,000 plaques/plate (15 cm) using Y 1090R$^-$ cells. Impressions were taken using nylon membranes, which were denatured, baked at 80°C for 2 hr, and prehybridized for 2 hr at 37°C in 50% deionized formamide, 1% sodium dodecyl sulfate, 1 M NaCl, and 100 μg/ml sonicated and boiled herring sperm DNA. The cDNA probe was added (approximately 5 x 10^6 dpm/ml) and allowed to hybridize with the membrane overnight at 37°C. The membranes were then washed at 45°C for 1 hr in 1 x standard saline citrate 150 mM NaCl/0· 1% sodium dodecyl sulfate and placed in cassettes for overnight exposure to Kodak XAR film at -70°C.

Seven related clones were identified by PCR and restriction enzyme analysis. These were assigned to three groups according to size and one member of each group was subcloned into the EcoRI site of p bluescript (Stratagene). Nucleotide sequences were determined by using the dideoxy-chain termination method (Sequenase: United States Biochemical). One clone contained a complete open reading frame of 1074 nucleotides. A plasmid for expressing this clone in eukaryotic cells was prepared as previously described [13]. COS-7 cells were transfected with the clone using DEAE-dextran and DMSO shock [20].

In order to perform radioligand binding studies, the transfected COS-7 cells were harvested after 3 days and membranes were prepared [13], resuspended at a 1 mg/ml concentration in 50 mM TRIS-HCl, 10 mM $MgCl_2$, 1 mM EDTA at pH 7.4, and frozen at -80° C. Thawed plasma membranes at a 0.5 mg/ml concentration were employed for radioligand binding competition studies. The binding of 5 nM [^{3}H]-PGE_2 (specific activity 183 Ci/mmol; Amersham) was determined in duplicate in a 200 μl volume at 25° C for 30 min. Binding reactions were terminated by the addition of 4 ml of ice-cold 50 mM TRIS-HCl at pH 7.4, followed by rapid filtration through Whatman GF/B filters and three additional 4 ml washes using a cell harvester (Brandel). Non-specific binding was determined by 10 μM PGE_2. All experiments were replicated three times.

cAMP studies involved splitting the cells 24 hr after transfection into 24 well plates and culturing for two more days in DMEM containing 5% calf serum. The cells were then rinsed with DMEM and preincubated for 1 min at 37°C with 400 µl/well DMEM containing 100 µg/ml isobutylmethylxanthine. The cells were then incubated for a further 3 min following the addition of 100 µl /DMEM containing isobutylmethylxanthine and the desired final drug concentration. The medium was removed and the cells were scraped in 150 µl/well ice cold 50 mM TRIS, 4 mM EDTA at pH 7.5 and placed on ice. The cell suspensions were transferred to microcentrifuge tubes, boiled for 5 min, and frozen at -20°C. The effects of PGE_2 and its analogs on cAMP formation were determined using a cAMP binding assay using protein kinase A, as previously described [13].

Intraocular pressure (IOP) studies were performed in female cynomolgus monkeys, weighing 2-4 kg. Cynomolgus monkeys were trained to accept pneumatonometry while conscious and were restrained in custom designed chairs. Both ocular normotensive monkeys and monkeys rendered unilaterally ocular hypertensive by circumferential argon laser treatment of the trabecular meshwork were used in these studies. Intraocular pressure was measured by means of an applanation pneumatonometer. One min before pneumatonometry, 25 µl of proparacaine (Allergan, Irvine, CA) was applied to minimize ocular discomfort during the pneumatonometric procedure. AH 13205 was administered to one eye in a 25 µl volume, an equal volume of vehicle was applied to the contralateral eye as a control. AH 13205 was administered twice daily with a 6 hr interval between doses in the 5 day studies in ocular normotensive monkeys. Intraocular pressure was recorded just before the first dose and then at 2,4, and 6 hr thereafter, the final reading just preceding the second daily dose. One day studies in ocular hypertensive monkeys involved only a single administration of AH 13205 to the ocular hypertensive eye. Intraocular pressure was recorded at 1 hr before and immediately prior to dosing and then at 1, 2, 4 and 6 hr post-dosing.

Aqueous flow was measured fluorphotometrically with a fluorotron instrument (Coherent) in female cynomolgus monkeys weighting 2-4 kg. The animals were sedated with ketamine, 7-10 mg/kg I.M. prior to each fluorphotometric scan. Background autofluorescence scans were taken on the afternoon prior to the fluorphotometry experiment. The experiment was commenced by giving one drop of 0.5% proparacaine (Ophthetic, Allergan) to each eye in order to increase corneal permeability. 5 min later a further drop of ophthetic was given. The eyelids were held open by speculums and 2 µl Na fluorescein (2%) drops were applied to the center of the cornea. This ophthetic/fluorescein dosing procedure was repeated four times at five min intervals and excess fluorescein was removed. This procedure "loaded" the eye with fluorescein and provided steady-state conditions for the experiment on the following day. Fluorphotometric measurements were performed 7 times, one hour apart, on the following day. Each eye was scanned at least twice per reading. Ketamine (7-10 mg/kg) was injected intramuscularly 5 min before each scan. Ketamine was used to sedate the animals and align the eyes with the fluorphotometric beam.

In order to calculate aqueous humor flow, values for anterior chamber depth and corneal thickness and curvature were obtained. Anterior chamber depth and corneal thickness were determined by pachymetry using a Haag-Streit slit lamp. Corneal curvature was determined using a keratometer. These values allowed anterior chamber volume to be calculated. Aqueous humor flow was obtained by software written for the IBM or Macintosh by P.L. Kaufman (University of Wisconsin) according to equations developed by Yablonski, et al. [21].

For aqueous humor flow studies with AH 13205, the drug was administered twice on the day before the flow experiment with a 6 hr interval between doses and again on the morning of the day of the flow experiment. In a different series of experiments, Timolol was employed as a reference drug, and was administered once on the day of the flow experiment.

All prostanoids were purchased from Cayman Chemical (Kalamazoo, MI), with the following exceptions. BW 245C was a gift from Burroughs Wellcome (Beckenham, UK); AH 13205 and AH 23848B were gifts from Glaxo (Ware, UK). Butaparost and AY 23626 were obtained from the Allergan Synthetic Chemistry Department. MB 28767 was a gift from Rhone-Poulenc (Dagenham, UK) and sulprostone was a gift from Berlex (Cedar Knolls, NJ).

Radiolabeled PGE_2 [5,6,8,9,11,12,14,15-^{3}H(N)] was purchased from New England Nuclear (Wilmington, DE).

All solutions for radioligand binding studies were prepared in TRIS-HCl buffer. AH 13205 was dissolved in 1% polysorbate 80/10 mM TRIS base for the intraocular pressure studies.

RESULTS

The human placenta cDNA encodes a protein of 358 amino acids with a molecular weight of 39,380. Hydropathy analysis and consensus with other cloned PG receptors indicate seven hydrophobic regions that are putative membrane-spanning domains of the receptor. In the

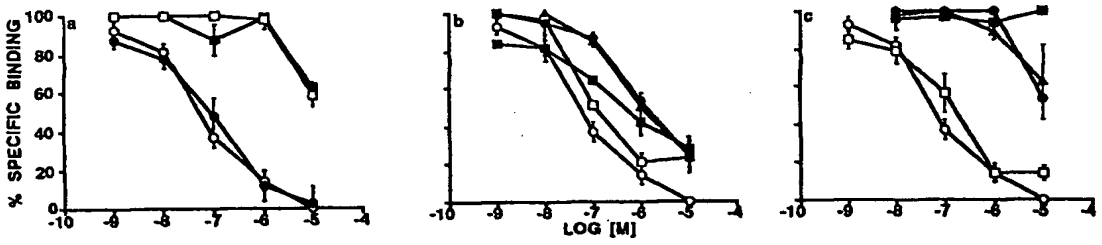

FIGURE 1 Competition by PGs with the radioligand binding of [^{3}H]PGE$_2$ to membranes prepared from COS-7 cells transfected with cDNA that encodes the human EP$_2$ receptor. A, Naturally occurring PGs. O, PGE$_2$; ●, PGE$_1$; □, PGD$_2$; ■, PGF$_{2\alpha}$, B, Reported EP$_2$-selective PGs. ▲, AH 13205; ■, butaprost; ●, 19(R)-OH-PGE$_2$; □, 11-deoxy-PGE$_1$. C, Other PGs. □, 16,16-Dimethyl-PGE$_2$; ▲, MB28767; ■, sulprostone; ●, 1-OH-PGE$_1$. In B and C the data from A for PGE$_2$ (O) are added for comparison. Data are the means ± standard errors of three separate experiments performed in duplicate. Reprinted by permission from Regan et al. Mol. Pharmacol. 46:213-220, 1994.

hydrophilic regions there are four consensus sites for N-linked glycosylation, two in the amino terminus, one in the first extracellular loop and one in the third extracellular loop. Within the proposed transmembrane domains, the amino acid identity with other cloned PG receptors was as follows: EP$_1$ 37%, butaprost-insensitive EP$_2$ 38%; EP$_{3A}$ 34%; TP 31%, FP 31%.

Pharmacological identity was determined by radioligand binding studies on membranes prepared from transfected COS-7 cells. The ability of unlabeled PGs and their synthetic analogs to compete with [^{3}H]-PGE$_2$ is illustrated in figure 1. PGE$_1$ and PGE$_2$ were potent competitors whereas PGF$_{2\alpha}$ and PGD$_2$ did not inhibit [^{3}H]-PGE$_2$ binding until a 10 μM concentration was achieved (Fig. 1a). PGE analogs with reported selectivity for the EP$_2$ receptor all competed with [^{3}H]-PGE$_2$ for the receptor with the following potency rank order 11-doexy PGE$_1$ > butaprost > AH 13205 = 19(R)-OH PGE$_2$ (Fig. 1b). The EP$_1$/EP$_3$ agonist sulprostone had no affinity over the concentration range employed and MB 28767 and PGE$_1$-1-OH competed with [^{3}H]-PGE$_2$ only at a 10 μM concentration (Fig. 1c). 16,16-dimethyl PGE$_2$ and PGE$_2$ were essentially equipotent (Fig. 1c).

Radioligand binding studies with additional PG analogs and other eicosanoids are depicted in Fig. 2. Radioligand binding studies with prostanoids that were believed not to exert their activity by interacting with EP$_2$ receptors provided no surprising results and experiments were confined to a

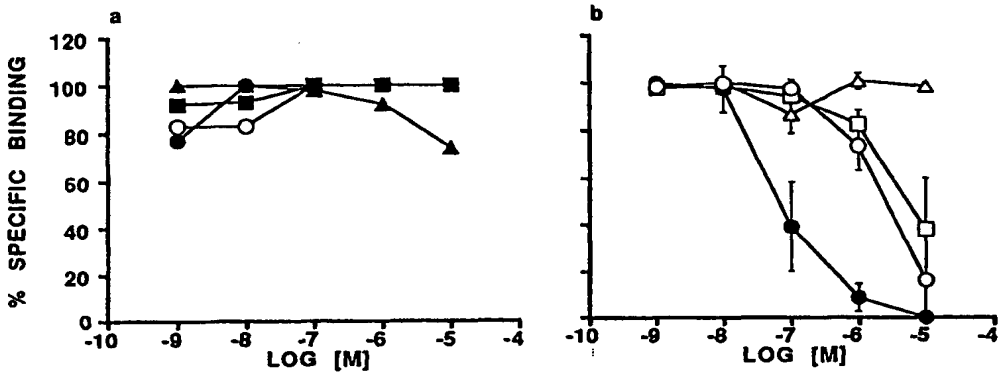

FIGURE 2 Competition by PGs with the radioligand binding of [^{3}H]PGE$_2$ to membranes prepared from COS-7 cells transfected with cDNA that encodes the human EP$_2$ receptor. A. 8-epi PGF$_{2\alpha}$ (■), PGJ$_2$ (▲), iloprost (O), 6-keto PGF$_{1\alpha}$ (●). B. AY 23626 (●), AH 23848B (Δ), BW 245C (□), 15-keto PGE$_2$ (O). Data are means ± standard errors.

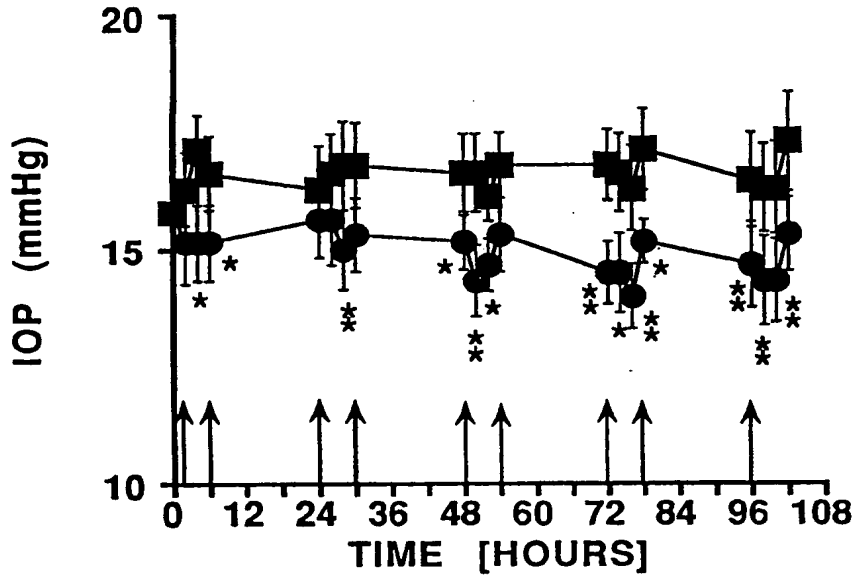

FIGURE 3. Effect of b.i.d. dosing with 0.1% AH 13205 for five consecutive days on normotensive monkey intraocular pressure. Eyes that received AH 13205 are represented by (●); eyes that received vehicle are represented by (■). Values are mean ± standard errors. Vertical arrows indicate times of topical drug administration. n=6; *p <0.05, **p <0.01.

single study in triplicate. Thus, iloprost, the prostacyclin metabolite 6-keto $PGF_{1\alpha}$, 8-epi $PGF_{2\alpha}$, and PGJ_2 exhibited no meaningful interaction with the human EP_2 receptor (Fig. 2a). The DP agonist BW 245C and the EP receptor ligands AH 23848B, AY 23626, and 15-keto PGE_2 were studied in duplicate in three separate experiments. Only AY 23626 potently competed with PGE_2 for the EP_2 receptor binding site (Fig. 2b).

The effect of AH 13205, at a 0.1% concentration given twice daily, on the intraocular pressure of normal monkeys is depicted in Fig. 3. Only a modest reduction in intraocular pressure was apparent during the first two study days. Thereafter, the ocular hypotensive activity of AH 13205 was more pronounced and a 2-3 mm Hg decrease in intraocular pressure was achieved. The ocular hypotensive activity was well-maintained over each 24 hr experimental cycle. The effect of AH 13205 in laser-induced ocular hypertensive monkeys is shown in Fig. 4. Similar reductions in intraocular pressure were produced by 0.1% (Fig. 4a) and 0.5% (Fig. 4b) doses of AH 13205.

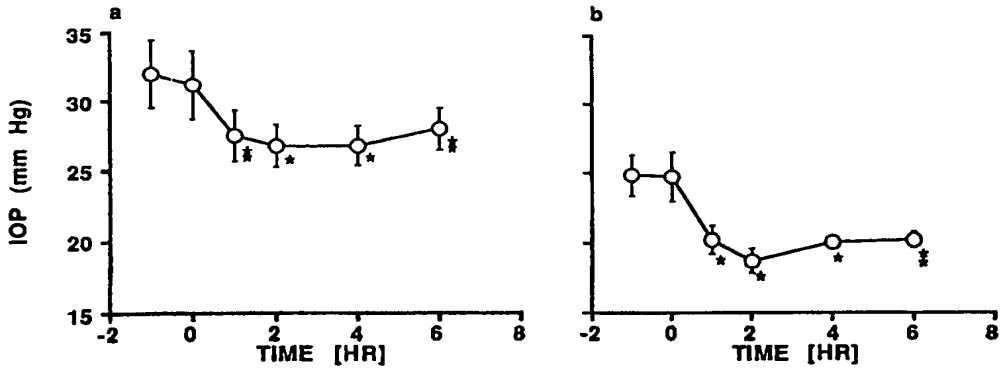

FIGURE 4. Effect of single doses of 0.1% (panel a) and 0.5% (panel b) AH 13205 on the intraocular pressure of laser-induced ocular hypertensive monkeys. Values are mean ± standard errors. n=6; *p <0.05, **p <0.01.

The effect of 0.1% AH 13205 on aqueous humor flow in ocular normotensive monkeys is described in Table 1. No significant effect on aqueous humor flow was apparent, aqueous humor flow increased by 0.26 µl/min at 2-4 hr after AH 13205 administration. Timolol produced a marked and statistically significant reduction in aqueous humor flow > 40% (Table 1).

Table 1 Effect of AH 13205 (0.1%) and Timolol (0.5%) compared to vehicle on aqueous humor flow (µL/min) in cynomolgus monkeys. Values are mean ± standard errors *p <0.05 according to Students' unpaired, two-tailed t test, n=6.

	Timolol 0.5%	Vehicle	AH 13205 0.1%
Aqueous Humor Flow (µl/min)-before drug	1.64 ± 0.14	1.90 ± 0.19	1.46 ± 0.06
Aqueous Humor Flow (µl/min)-after drug	0.91 ± 0.09	1.72 ± 0.17	1.72 ± 0.17
% Change in Aqueous Humor Flow	-41.8 ± 8.0	-7.0 ± *12.3	+18.7 ± 12.0

DISCUSSION

The receptor reported herein has the functional and radioligand binding characteristics that are consistent with the pharmacologically defined prostanoid EP_2 receptor. Thus, the selective EP_2 receptor agonists butaprost [3] and AH 13205 [3,19] compete with PGE_2 for the receptor binding sites and stimulate cAMP in cells transfected with this receptor [13]. We have extended our initial findings by examining the binding characteristics of additional natural prostanoids and selected synthetic analogs. These provide confirmatory evidence for EP_2 receptor pharmacology. Thus, the high affinity of AY 23626 and the marked loss in activity associated with 15-keto PGE_2, compared to PGE_2, are consistent with EP_2 receptor pharmacology [22]. BW 245C was also studied since it has been previously reported to stimulate both a DP receptor and an additional prostanoid receptor associated with vascular smooth muscle [23]. The EP_2 receptor was, in the absence of evidence to the contrary, proposed as the possible additional BW 245C sensitive receptor present in the rabbit jugular vein [23]. The results of radioligand binding studies in COS-7 cells transfected with the human EP_2 receptor suggest that BW 245C has no affinity for the EP_2 receptor until a concentration of 1 µM is exceeded. The identity of the additional BW 245C receptor in the rabbit jugular vein remains, therefore, uncertain. The human EP_2 receptor is differentiated from the recently proposed EP_4 receptor [4] since the EP_4 antagonist did not compete with PGE_2 at concentrations up to and including 10 µM. Finally, the IP receptor agonist iloprost, the PGI_2 metabolite 6-keto $PGF_{1\alpha}$, 8-epi $PGF_{2\alpha}$ and the PGD_2 metabolite PGJ_2 exhibited no meaningful affinity for the EP_2 receptor.

The pharmacological definition of the cloned receptor previously suggested as EP_2 [10,17] remains to be determined. Four EP receptors have been reported according to pharmacological criteria [3] and only the EP_4 receptor remains to be assigned a molecular structural identity. In the absence of published data which provides adequate pharmacological characterization of the cloned receptor previously suggested as EP_2, it seems premature to designate it as EP_4.

Studies with the selective agonist AH 13205 indicate that EP_2 receptor stimulation results in a decrease in primate intraocular pressure. These findings with AH 13205 add to the list of prostanoid receptors that represent potential targets for designing ocular hypotensive drugs. FP receptor and EP_3 receptor agonists have already been described as potent ocular hypotensive agents in monkeys [18]. Moreover, the DP receptor agonist BW 245C is a potent ocular hypotensive in laser-induced ocular hypertensive monkeys [24] and has effects in human volunteers [25]. The involvement of EP_1, EP_4 and IP receptors in mediating ocular hypotension in primates has yet to be demonstrated and cannot be dismissed at this time. Comparing the activity of AH 13205 with that of the FP agonist 17-phenyl $PGF_{2\alpha}$ and the EP_3 agonist MB 28767 [18], it appears that EP_3 and FP agonists produced a greater degree of ocular hypotension. The reason for this remains to be determined. AH 13205 does not inhibit aqueous humor secretion and consequently, the lack of profound ocular

hypotensive effect in normal monkeys cannot be ascribed to AH 13205 behaving as an 'inflow' drug. Disruption of the blood-aqueous barrier by prostanoids has been described as an EP_2 receptor mediated phenomenon [26]. If blood-aqueous barrier disruption occurred in response to EP_2 receptor stimulation in primates, this could physiologically antagonize the ocular hypotensive activity.

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Reference 5

US005877211A

United States Patent [19]

Woodward

[11] Patent Number: 5,877,211

[45] Date of Patent: Mar. 2, 1999

[54] **EP_2 RECEPTOR AGONISTS AS NEUROPROTECTIVE AGENTS FOR THE EYE**

[75] Inventor: **David F. Woodward**, Lake Forest, Calif.

[73] Assignee: **Allergan**, Irvine, Calif.

[21] Appl. No.: **975,314**

[22] Filed: **Nov. 21, 1997**

[51] **Int. Cl.**[6] **A61K 31/215**

[52] **U.S. Cl.** **514/530**; 514/573; 514/912

[58] **Field of Search** 514/530, 573, 514/912

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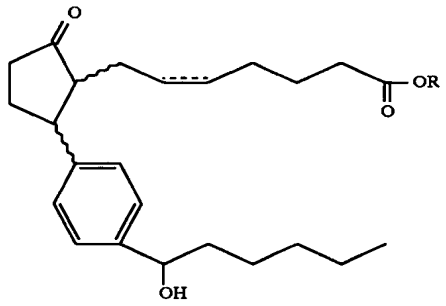
Primary Examiner—Zohreh Fay

Attorney, Agent, or Firm—Robert J. Baren; Martin A. Voet; James M. Hoch

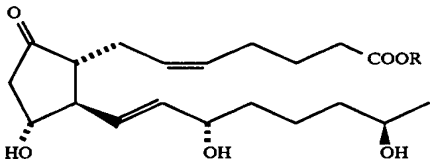
[57] **ABSTRACT**

The invention relates to the use of EP_2 receptor agonists as neuroprotective agents. In particular said compounds are represented by the formulae:

I



III



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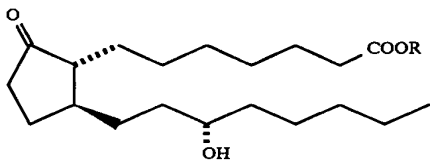

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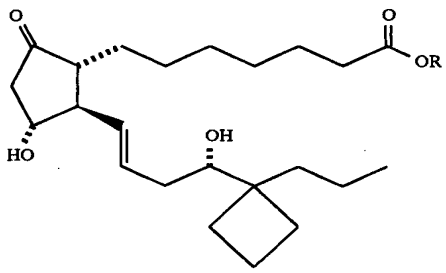


wherein the broken line attachment to the cyclopentane ring or the omega chain indicates the α configuration and the solid line attachment to the cyclopentane ring or the omega chain indicates the β configuration, R is hydrogen or a saturated or unsaturated acyclic hydrocarbon group having from 1 to about 20 carbon atoms, or $—(CH_2)_mR_1$ wherein m is 0–10, and R_1 is an aliphatic ring having from about 3 to about 7 carbon atoms, or an aryl or heteroaryl ring having from about 4 to about 10 carbon atoms and wherein the heteroatom is selected from the group consisting of N, O and S.

17 Claims, 2 Drawing Sheets

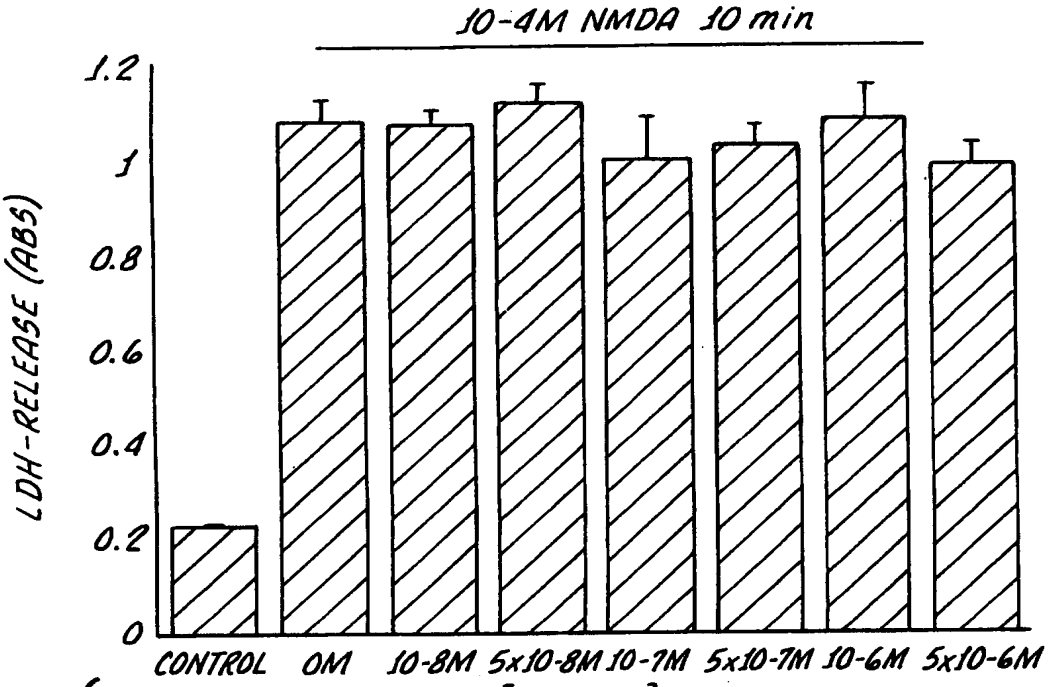
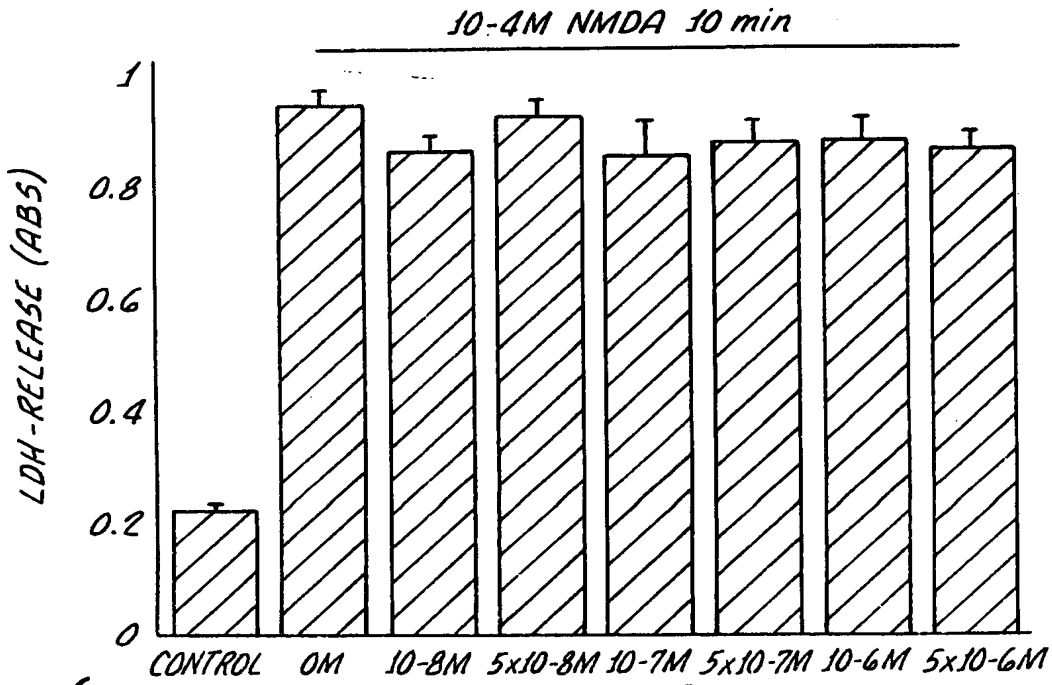

10-4M NMDA 10 min
LDH-RELEASE (ABS)
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
OM
10-8M
5x10-8M
10-7M
5x10-7M
10-6M
5x10-6M
[PGF2α]

FIG. 1A.


10-4M NMDA 10 min
LDH-RELEASE (ABS)
1
0.8
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CONTROL
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10-8M
5x10-8M
10-7M
5x10-7M
10-6M
5x10-6M
[PGF2α-1-OH]

FIG. 1B.

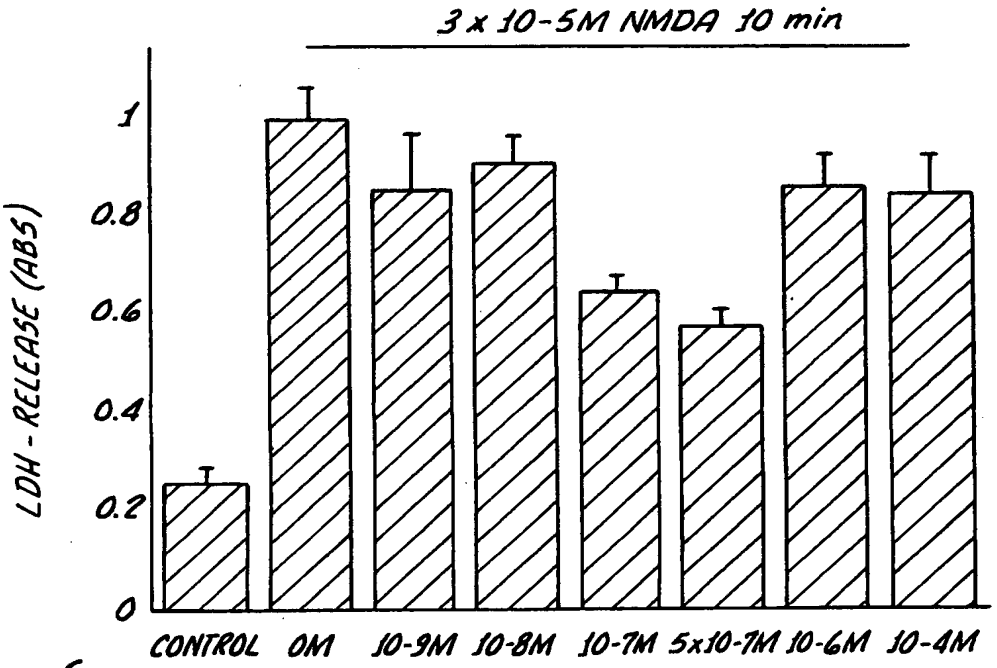

3 x 10-5M NMDA 10 min
LDH - RELEASE (ABS)
1
0.8
0.6
0.4
0.2
0
CONTROL
OM
10-9M
10-8M
10-7M
5x10-7M
10-6M
10-4M
[AH 13205]

FIG. 1C.

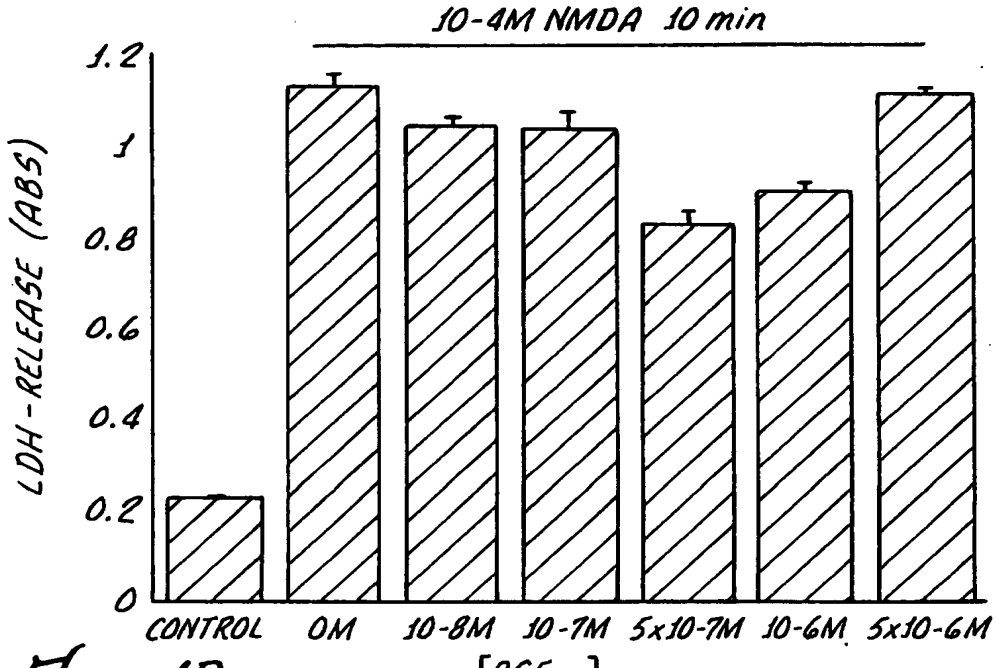

10-4M NMDA 10 min
LDH - RELEASE (ABS)
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
OM
10-8M
10-7M
5x10-7M
10-6M
5x10-6M
[PGE2]

FIG. 1D.

EP_2 RECEPTOR AGONISTS AS NEUROPROTECTIVE AGENTS FOR THE EYE

FIELD OF THE INVENTION

The present invention relates to the use of EP_2 receptor agonists to provide a neuroprotective effect to the eye of a mammal.

BACKGROUND OF THE INVENTION

Ocular hypotensive agents are useful in the treatment of a number of various ocular hypertensive conditions, such as post-surgical and post-laser trabeculectomy ocular hypertensive episodes, glaucoma, and as presurgical adjuncts.

Glaucoma is a disease of the eye characterized by increased intraocular pressure. On the basis of its etiology, glaucoma has been classified as primary or secondary. For example, primary glaucoma in adults (congenital glaucoma) may be either open-angle or acute or chronic angle-closure. Secondary glaucoma results from pre-existing ocular diseases such as uveitis, intraocular tumor or an enlarged cataract.

The underlying causes of primary glaucoma are not yet known. The increased intraocular tension is due to the obstruction of aqueous humor outflow. In chronic open-angle glaucoma, the anterior chamber and its anatomic structures appear normal, but drainage of the aqueous humor is impeded. In acute or chronic angle-closure glaucoma, the anterior chamber is shallow, the filtration angle is narrowed, and the iris may obstruct the trabecular meshwork at the entrance of the canal of Schlemm. Dilation of the pupil may push the root of the iris forward against the angle, and may produce pupillary block and thus precipitate an acute attack. Eyes with narrow anterior chamber angles are predisposed to acute angle-closure glaucoma attacks of various degrees of severity.

Secondary glaucoma is caused by any interference with the flow of aqueous humor from the posterior chamber into the anterior chamber and subsequently, into the canal of Schlemm. Inflammatory disease of the anterior segment may prevent aqueous escape by causing complete posterior synechia in iris bombe and may plug the drainage channel with exudates. Other common causes are intraocular tumors, enlarged cataracts, central retinal vein occlusion, trauma to the eye, operative procedures and intraocular hemorrhage.

Considering all types together, glaucoma occurs in about 2% of all persons over the age of 40 and may be asymptotic for years before progressing to rapid loss of vision. In cases where surgery is not indicated, topical β-adrenoreceptor antagonists have traditionally been the drugs of choice for treating glaucoma.

It has long been known that one of the sequelae of glaucoma is damage to the optic nerve head. This damage, referred to as "cupping", results in depressions in areas of the nerve fiber of the optic disk. Loss of sight from this cupping is progressive and can lead to blindness if the condition is not treated effectively.

Unfortunately lowering intraocular pressure by administration of drugs or by surgery to facilitate outflow of the aqueous humor is not always effective in obviating damage to the nerves in glaucomatous conditions. This apparent contradiction is addressed by Cioffi and Van Buskirk [*Surv. of Ophthalmol.*, 38, Suppl. p. S107–16, discussion S116–17, May 1994] in the article, "Microvasculature of the Anterior Optic Nerve". The abstract states:

> The traditional definition of glaucoma as a disorder of increased intraocular pressure (IOP) oversimplifies the clinical situation. Some glaucoma patients never have higher than normal IOP and others continue to develop optic nerve damage despite maximal lowering of IOP. Another possible factor in the etiology of glaucoma may be regulation of the regional microvasculature of the anterior optic nerve. One reason to believe that microvascular factors are important is that many microvascular diseases are associated with glaucomatous optic neuropathy.

Subsequent to Cioffi, et al., Matusi published a paper on the "Ophthalmologic aspects of Systemic Vasculitis" [*Nippon Rinsho,* 52 (8), p. 2158–63, August 1994] and added further support to the assertion that many microvascular diseases are associated with glaucomatous optic neuropathy. The summary states:

> Ocular findings of systemic vasculitis, such as polyarteritis nodosa, giant cell angitis and aortitis syndrome were reviewed. Systemic lupus erythematosus is not categorized as systemic vasculitis, however its ocular findings are microangiopathic. Therefore, review of its ocular findings was included in this paper. The most common fundus finding in these diseases is ischemic optic neuropathy or retinal vascular occlusions. Therefore several points in diagnosis or pathogenesis of optic neuropathy and retinal and choroidal vaso-occlusion were discussed. Choroidal ischemia has come to be able to be diagnosed clinically, since fluorescein angiography was applied in these lesions. When choroidal arteries are occluded, overlying retinal pigment epithelium is damaged. This causes disruption of barrier function of the epithelium and allows fluid from choroidal vasculatures to pass into subsensory retinal spaces. This is a pathogenesis of serous detachment of the retina. The retinal arterial occlusion formed nonperfused retina. Such hypoxic retina released angiogenesis factors which stimulate retinal and iris neovascularizations and iris neovascularizations may cause neovascular glaucoma.

B. Schwartz, in "Circulatory Defects of the Optic Disk and Retina in Ocular Hypertension and High Pressure Open-Angle Glaucoma" [*Surv. Ophthalmol.*, 38, Suppl. pp. S23–24, May 1994] discusses the measurement of progressive defects in the optic nerve and retina associated with the progression of glaucoma. He states:

> Fluorescein defects are significantly correlated with visual field loss and retinal nerve fiber layer loss. The second circulatory defect is a decrease of flow of fluorescein in the retinal vessels, especially the retinal veins, so that the greater the age, diastolic blood pressure, ocular pressure and visual field loss, the less the flow. Both the optic disk and retinal circulation defects occur in untreated ocular hypertensive eyes. These observations indicate that circulatory defects in the optic disk and retina occur in ocular hypertension and open-angle glaucoma and increase with the progression of the disease.

Thus, it is evident that there is an unmet need for agents that have neuroprotective effects in the eye that can stop or retard the progressive damage that occurs to the nerves as a result of glaucoma or other ocular afflictions.

Prostaglandins were earlier regarded as potent ocular hypertensives; however, evidence accumulated in the last two decades shows that some prostaglandins are highly effective ocular hypotensive agents and are ideally suited for the long-term medical management of glaucoma. (See, for

example, Starr, M. S. *Exp. Eye Res.* 1971, 11, pp. 170–177; Bito, L. Z *Biological Protection with Prostaglandins* Cohen, M. M., ed., Boca Raton, Fla., CRC Press Inc., 1985, pp. 231–252; and Bito, L. Z., *Applied Pharmacology in the Medical Treatment of Glaucomas* Drance, S. M. and Neufeld, A. H. eds., New York, Grune & Stratton, 1984, pp. 477–505). Such prostaglandins include $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_2, and certain lipid-soluble esters, such as C_1 to C_5 alkyl esters, e.g. 1-isopropyl ester, of such compounds.

In the U.S. Pat. No. 4,599,353 certain prostaglandins, in particular PGE_2 and $PGF_{2\alpha}$ and the C_1 to C_5 alkyl esters of the latter compound, were reported to possess ocular hypotensive activity and were recommended for use in glaucoma management.

Although the precise mechanism is not yet known, recent experimental results indicate that the prostaglandin-induced reduction in intraocular pressure results from increased uveoscleral outflow [Nilsson et al., *Invest. Ophthalmol. Vis. Sci.* 2 8(suppl), 284 (1987)].

The isopropyl ester of $PGF_{2\alpha}$ has been shown to have significantly greater hypotensive potency than the parent compound, which was attributed to its more effective penetration through the cornea. In 1987 this compound was described as "the most potent ocular hypotensive agent ever reported." [See, for example, Bito, L. Z., *Arch. Ophthalmol.* 105, 1036 (1987), and Siebold et al., *Prodrug* 5, 3 (1989)].

Whereas prostaglandins appear to be devoid of significant intraocular side effects, ocular surface (conjunctival) hyperemia and foreign-body sensation have been consistently associated with the topical ocular use of such compounds, in particular $PGF_{2\alpha}$ and its prodrugs, e.g. its 1-isopropyl ester, in humans. The clinical potential of prostaglandins in the management of conditions associated with increased ocular pressure, e.g. glaucoma, is greatly limited by these side effects.

Certain phenyl and phenoxy mono, tri and tetra nor prostaglandins and their 1-esters are disclosed in European Patent Application 0,364,417 as useful in the treatment of glaucoma or ocular hypertension.

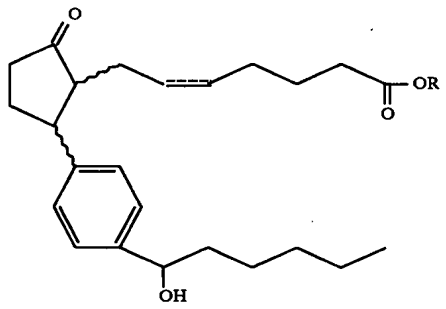
In a series of co-pending United States patent applications assigned to Allergan, Inc. prostaglandin esters with increased ocular hypotensive activity accompanied by no or substantially reduced side-effects are disclosed. The co-pending U.S. Ser. No. 386,835 (filed 27 Jul. 1989), relates to certain 11-acyl-prostaglandins, such as 11-pivaloyl, 11-acetyl, 11-isobutyryl, 11-valeryl, and 11-isovaleryl $PGF_{2\alpha}$. Intraocular pressure reducing 15-acyl prostaglandins are disclosed in the co-pending application U.S. Ser. No. 357,394 (filed 25 May 1989). Similarly, 11,15-9,15- and 9,11-diesters of prostaglandins, for example 11,15-dipivaloyl $PGF_{2\alpha}$ are known to have ocular hypotensive activity. See the co-pending patent applications U.S. Ser. No. 385,645 filed 27 Jul. 1990, now U.S. Pat. No. 4,494,274; U.S. Ser. No. 584,370 which is a continuation of U.S. Ser. Nos. 386,312, and 585,284, now U.S. Pat. No. 5,034,413 which is a continuation of U.S. Ser. No. 386,834, where the parent applications were filed on 27 Jul. 1989. The disclosures of these patent applications are hereby expressly incorporated by reference.

Finally, certain EP_2-receptor agonists are disclosed in Nials et al, *Cardiovascular Drug Reviews*, Vol. 11, No. 2, pp. 165–179, Coleman et al, *Comprehensive Medicinal Chemistry*, Vol. 3, pp. 643–714, 1990 and Woodward et al, *Prostaglandins*, pp. 371–383, 1993.

SUMMARY OF THE INVENTION

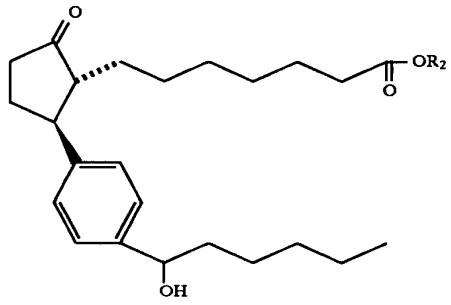
We have found that EP_2-receptor agonists are potent neuroprotective agents. We have further found that (±) trans-2-[-4(1-hydroxyhexyl)phenyl]-5-oxocyclopentaneheptanoicacid, and certain other EP_2-receptor agonists, described below, and ester and unsaturated derivatives thereof, are especially useful in providing a neuroprotective effect to the eye of a mammal, e.g. a human.

The present invention relates to methods of providing a neuroprotective effect to the eye of a mammal, e.g. a human, which comprises administering an effective amount of a compound represented by the formula I



wherein, the wavy bands indicate the α or β configuration, R is hydrogen or a saturated or unsaturated acyclic hydrocarbon group having from 1 to about 20 carbon atoms, or $-(CH_2)_mR_1$ wherein m is 0–10, and R_1 is an aliphatic ring having from about 3 to about 7 carbon atoms, or an aryl or heteroaryl ring having from about 4 to about 10 carbon atoms, e.g. R_1 may be cyclohexyl, phenyl, thienyl, pyridyl or furanyl, or a pharmaceutically acceptable salt thereof and the dashed bond represents either a single or double bond which may be in the cis or trans position. Preferably R_1 is lower alkyl.

More preferably the method of the present invention comprises administering a compound represented by the formula II



wherein R_2 is hydrogen or a lower alkyl radical and the other symbols are as defined above.

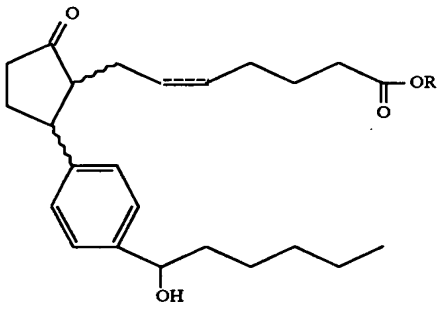
BRIEF DESCRIPTION OF THE DRAWING FIGURES

FIGS. 1A through 1D show a comparison of the neuroprotective effects of EP_2 agonists, e.g. see FIGS. 1C and 1D, with prostaglandins having no EP_2 agonist activity, e.g. see FIGS. 1A and 1B, in preventing neuronal damage.

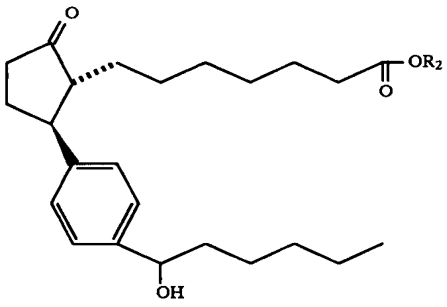
DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of (±) trans-2-[-4(1-hydroxyhexyl)phenyl]-5-oxocyclopentaneheptanoicacid, and ester and unsaturated

derivatives thereof as neuroprotective agents. These therapeutic agents are represented by compounds having the formula I



as defined above. The preferred compounds used in accordance with the present invention are encompassed by the following structural formula II



wherein R_2 is hydrogen or a lower alkyl radical.

In all of the above formulae, as well as in those provided hereinafter, the straight lines represent bonds. Where there is no symbol for the atoms between the bonds, the appropriate carbon-containing radical is to be inferred. For example in formula I, the radical between the cyclopentyl ring and the

radical is a polymethylene (CH_2) radical, i.e. a hexylenyl radical. The dotted lines on the bond between carbons 5 and 6 (C-5), indicate a single or a double bond which can be in the cis or trans configuration. The radical adjacent to the double bond is a CH radical. If two solid lines are used that indicates a specific configuration for that double bond. Hatched lines at positions C-9 and C-11 indicate the α configuration. If one were to draw the β configuration, a solid triangular line would be used.

In the compounds used in accordance with the present invention, compounds having the C-9 or C-11 substituents in the α or β configuration are contemplated. In all formulas provided herein, broken line attachments to the cyclopentane ring or the omega chain indicate substituents in the α configuration. Thickened solid line attachments to the cyclopentane ring or the omega chain indicate substituents in the β configuration.

For the purpose of this invention, unless further limited, the term "alkyl" refers to alkyl groups having from one to ten carbon atoms and includes "lower alkyl" radicals having from one to five carbon atoms, the term "cycloalkyl" refers to cycloalkyl groups having from three to seven carbon atoms, the term "aryl" refers to aryl groups having from four to ten carbon atoms. The term "saturated or unsaturated acyclic hydrocarbon group" is used to refer to straight or branched chain, saturated or unsaturated hydrocarbon groups having from one to about six, preferably one to about four carbon atoms. Such groups include alkyl, alkenyl and alkynyl groups of appropriate lengths, and preferably are alkyl, e.g. methyl, ethyl, propyl, butyl, pentyl, or hexyl, or an isomeric form thereof.

The definition of R may include a cyclic component, $-(CH_2)_mR_1$, wherein m is 0–10, R_2 is an aliphatic ring from about 3 to about 7 carbon atoms, or an aromatic or heteroaromatic ring. The "aliphatic ring" may be saturated or unsaturated, and preferably is a saturated ring having 3–7 carbon atoms, inclusive. As an aromatic ring, R_1 preferably is phenyl, and the heteroaromatic rings have oxygen, nitrogen or sulfur as a heteroatom, i.e., R_1 may be thienyl, furanyl, pyridyl, etc. Preferably m is 0–4.

Preferred representatives of the compounds within the scope of the present invention are (±) trans-2-[-4(1-hydroxyhexyl)phenyl]-5-oxocyclopentaneheptanoicacid, unsaturated derivatives thereof, and lower alkyl esters of these compounds.

A compound which may be used in the pharmaceutical compositions and the methods of treatment of the present invention is (±) trans-2-[-4(1-hydroxyhexyl)phenyl]-5-oxocyclopentaneheptanoicacid.

A pharmaceutically acceptable salt is any salt which retains the activity of the parent compound and does not impart any deleterious or undesirable effect on the subject to whom it is administered and in the context in which it is administered. Such salts are those formed with pharmaceutically acceptable cations, e.g., alkali metals, alkali earth metals, etc.

Pharmaceutical compositions may be prepared by combining a therapeutically effective amount of at least one compound according to the present invention, or a pharmaceutically acceptable salt thereof, as an active ingredient, with conventional ophthalmically acceptable pharmaceutical excipients, and by preparation of unit dosage forms suitable for topical ocular use. The therapeutically efficient amount typically is between about 0.0001 and about 5% (w/v), preferably about 0.001 to about 1.0% (w/v) in liquid formulations.

For ophthalmic application, preferably solutions are prepared using a physiological saline solution as a major vehicle. The pH of such ophthalmic solutions should preferably be maintained between 4.5 and 8.0 with an appropriate buffer system, a neutral pH being preferred but not essential. The formulations may also contain conventional, pharmaceutically acceptable preservatives, stabilizers and surfactants.

Preferred preservatives that may be used in the pharmaceutical compositions of the present invention include, but are not limited to, benzalkonium chloride, chlorobutanol, thimerosal, phenylmercuric acetate and phenylmercuric nitrate. A preferred surfactant is, for example, Tween 80. Likewise, various preferred vehicles may be used in the ophthalmic preparations of the present invention. These vehicles include, but are not limited to, polyvinyl alcohol, povidone, hydroxypropyl methyl cellulose, poloxamers, carboxymethyl cellulose, hydroxyethyl cellulose cyclodextrin and purified water.

Tonicity adjustors may be added as needed or convenient. They include, but are not limited to, salts, particularly sodium chloride, potassium chloride, mannitol and glycerin, or any other suitable ophthalmically acceptable tonicity adjustor.

Various buffers and means for adjusting pH may be used so long as the resulting preparation is ophthalmically acceptable. Accordingly, buffers include acetate buffers, citrate buffers, phosphate buffers and borate buffers. Acids or bases may be used to adjust the pH of these formulations as needed.

In a similar vein, an ophthalmically acceptable antioxidant for use in the present invention includes, but is not limited to, sodium metabisulfite, sodium thiosulfate, acetylcysteine, butylated hydroxyanisole and butylated hydroxytoluene.

Other excipient components which may be included in the ophthalmic preparations are chelating agents. The preferred chelating agent is edentate disodium, although other chelating agents may also be used in place of or in conjunction with it.

The ingredients are usually used in the following amounts:

Ingredient	Amount (% w/v)
active ingredient	about 0.001–5
preservative	0–0.10
vehicle	0–40
tonicity adjustor	0–10
buffer	0.01–10
pH adjustor	q.s. pH 4.5–8.0
antioxidant	as needed
surfactant	as needed
purified water	as needed to make 100%

The actual dose of the active compounds of the present invention depends on the specific compound, and on the condition to be treated; the selection of the appropriate dose is well within the knowledge of the skilled artisan.

The ophthalmic formulations for use in the method of the present invention are conveniently packaged in forms suitable for metered application, such as in containers equipped with a dropper, to facilitate application to the eye. Containers suitable for dropwise application are usually made of suitable inert, non-toxic plastic material, and generally contain between about 0.5 and about 15 ml solution. One package may contain one or more unit doses.

Especially preservative-free solutions are often formulated in non-resealable containers containing up to about ten, preferably up to about five units doses, where a typical unit dose is from one to about 8 drops, preferably one to about 3 drops. The volume of one drop usually is about 20–35 μl.

The invention is further illustrated by the following non-limiting Examples.

EXAMPLE 1

(±) Trans-2-[-4(1-hydroxyhexyl)phenyl]-5-oxocyclo-pentaneheptanoicacid and Lower Alkyl Esters Thereof

The above acid compound is well known and may be purchased or synthesized by methods known in the art. The lower alkyl esters of this compound may be made by the esterification procedures described in the various patent applications described in the Background of the Invention.

EXAMPLE 2

Method of Measuring a Neuroprotective Effect

The dissection and dissociation of the rat hippocampal neuron cell cultures was carried out. Briefly, whole cerebral neocortices were removed from fetal rats, gestation age 15–19 days and kept in calcium-, magnesium-free Hanks' balanced salt solution. The hippocampi were removed under a dissecting microscope and the meninges were stripped away. When all the hippocampi were removed, the tissues were incubated in 0.05% trypsin solution for 30 minutes at 37° C. At the end of 340 minutes, the trypsin solution was replaced with plating medium (minimal essential medium supplemented with 2% Hyclone horse serum, 1% fetal calf serum, 25 mM glucose, 1% glutamine and 1% penicillin/streptomycin and N_2 supplement). Then the tissues were triturated with a Pasteur pipette 10 times and then again with a pipette whose tip has been fire polished to about half the normal diameter. The dissociated neuronal cells then were plated on poly D-lysine coated, 15 mm 24 well plates (2×10^5 cells/well) in plating medium.

The cell cultures were kept at 37° C. in a humidified, 5% CO_2-containing atmosphere. After 1–2 days, the horse serum level in the plating media was increased to 8%. After 4–7 days, the non-neuronal cell division was halted by 24 hours exposure to 10^{-6}M Cytosine arabinoside (ARA-C), and the cells were then placed into growing medium with 4% horse serum, 1% fetal calf serum, 25 mM glucose, 1% glutamine and 1% penicillin/streptomycin and N_2 supplement. Subsequent medium replacement was carried out every other day until the neuronal cells matured (15–20 days). Only matured cell cultures were selected for study.

Exposure of the excitatory amino acids was performed in minimal essential medium (MEM). Extreme care was taken to wash out the growing medium from cultures before the addition of the excitatory amino acid since the neurons are very sensitive to disturbance. Matured cell cultures were exposed to either glutamate, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), or kainic acid.

Cytotoxicity or cell injury was scored by light microscopy examination with trypan blue. In most experiments, the overall neuronal cell injury was quantitated by the amount of lactate dehydrogenase (LDH) released by the damaged cells into the media 24 hours after drug exposure.

LDH was measured at room temperature using Promega non-radioactive cytotoxicity assay kit. The absorbance of the reaction mixture was measured at 490 nm.

The Figure shows examples of LDH release after exposure of different exitotoxins and their antagonists.

As shown in the Figure, the effects of the EP_2 agonist AH 13205 on NMDA-induced neurotoxicity is shown in the top right panel. It is noted that $PGF_{2\alpha}$ and $PGF_{2\alpha}$ 1-OH are inactive, thereby indicating that this is a specific EP_2 receptor mediated effect.

EXAMPLE 3

Determination of EP_2 Receptor Activity

EP_2 receptor activity may be measured in accordance with the procedure disclosed in Woodward et al, *Prostaglandins*, pp. 371–383, 1993, which is hereby incorporated by reference in its entirety.

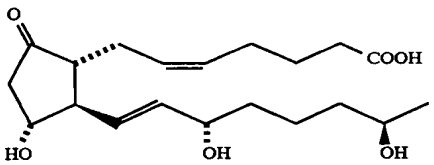
EXAMPLE 4

Method of Measuring a Neuroprotective Effect

The Experiment of Example 2 is repeated with other EP_2 Agonists, i.e. 19 (R)-OH PGE_2, AY 23626 and methyl 9-keto-11α,15α-dihydroxy-16,16 trimethyleneprosta-13-

transenoate (butaprost) and the results are essentially as shown in the Figure for AH 13205.

19 (R)-OH PGE_2, AY 23626 and butaprost have the following structures, respectively.



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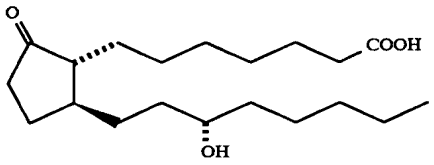

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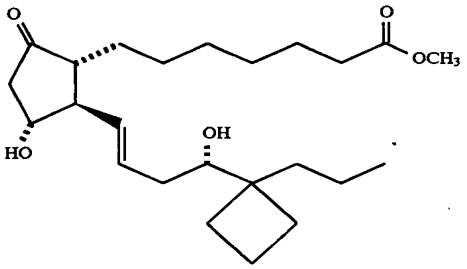


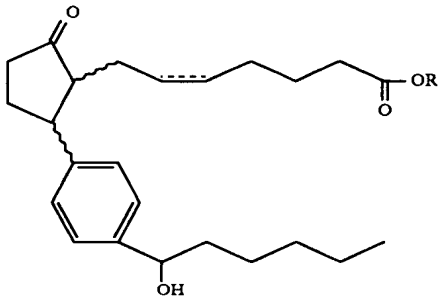
VIII

However, as with (±) trans-2-[-4(1-hydroxyhexyl)phenyl] -5-oxocyclopentaneheptanoicacid, the corresponding 1-ester or, in the case of butaprost other 1-ester derivatives as well as the 1-acid derivative are also useful in the method of the present invention.

The foregoing description details specific methods and compositions that can be employed to practice the present invention, and represents the best mode contemplated. However, it is apparent from one of ordinary skill in the art that different pharmaceutical compositions may be prepared and used with substantially the same results. That is, other EP_2-receptor agonists, will effectively lower intraocular pressure in animals and are within the broad scope of the present invention.

I claim:

1. A method of providing a neuroprotective effect to the eye of a mammal not having higher than normal intraocular pressure (IOP) which comprises applying to the eye an amount sufficient to treat ocular hypertension of a compound of formula I, III, IV or V



I

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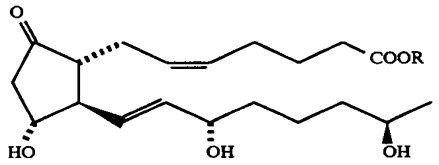

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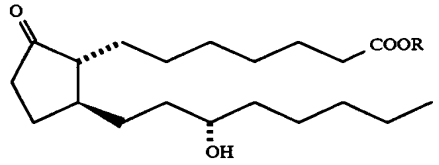

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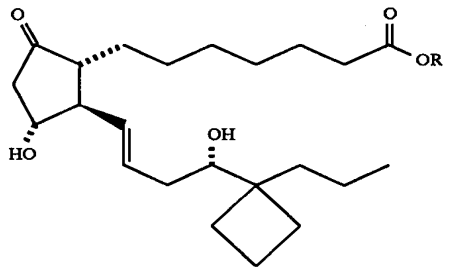
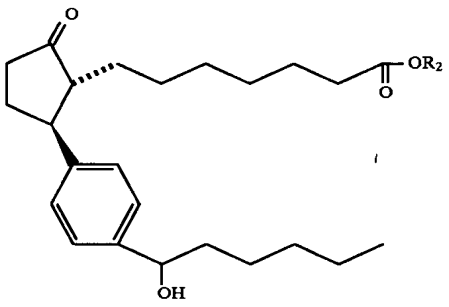


V

wherein the broken line attachment to the cyclopentane ring or the omega chain indicates the α configuration and the solid line attachment to the cyclopentane ring or the omega chain indicates the β configuration, R is hydrogen or a saturated or unsaturated acyclic hydrocarbon group having from 1 to about 20 carbon atoms, or $—(CH_2)_mR_1$ wherein m is 0–10, and R_1 is an aliphatic ring having from about 3 to about 7 carbon atoms, or an aryl or heteroaryl ring having from about 4 to about 10 carbon atoms and wherein the heteroatom is selected from the group consisting of N, O and S.

2. The method of claim 1 wherein said compound is a prostaglandin derivative of the formula II, VI, VII or VIII



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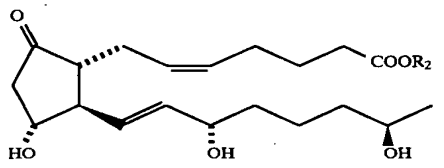

VI

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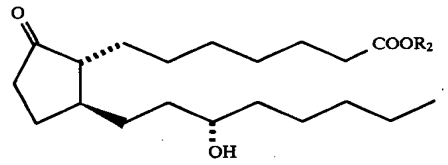
VII



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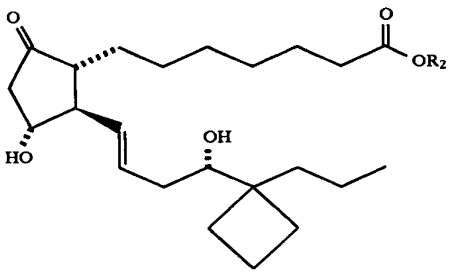
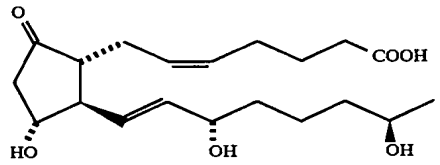


wherein R_2 is a hydrogen or a lower alkyl radical.

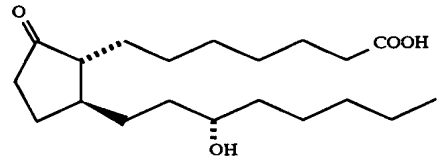
3. The method of claim 2 wherein said compound is

VI



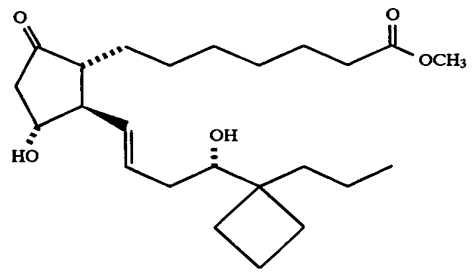
4. The method of claim 2 wherein said compound is

VII



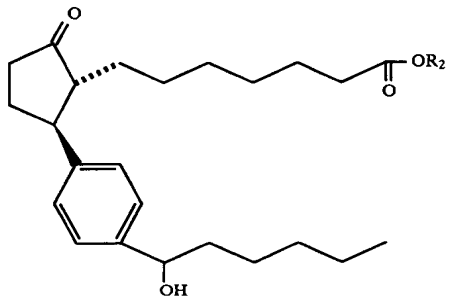
5. The method of claim 2 wherein said compound is

VIII



6. The method of claim 2 wherein said compound is

II

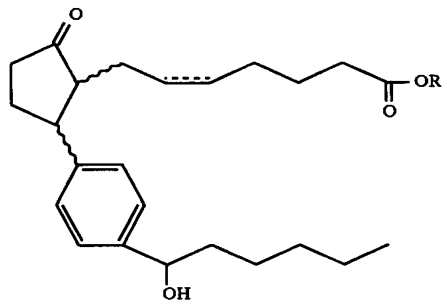


wherein R_2 is hydrogen or a lower alkyl radical.

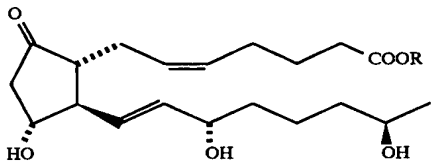
7. A method for providing a neuroprotective effect to the eye of a mammal not having higher than normal intraocular pressure (IOP) which comprises applying to the eye an amount sufficient to treat ocular hypertension of a compound having EP_2 receptor agonist activity.

8. A method of protecting the retinal or optic nerve cells in a mammal not having higher than normal intraocular pressure (IOP) suffering a noxious action or at risk of experiencing a noxious action on said nerve cells comprising administering to said mammal an effective amount of a compound of formula I, III, IV or V to inhibit or prevent nerve cell injury or death

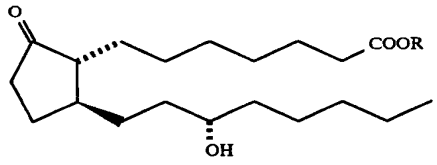
I



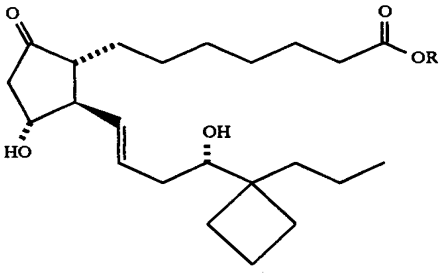
III



IV



V



wherein the broken line attachment to the cyclopentane ring or the omega chain indicates the α configuration and the solid line attachment to the cyclopentane ring or the omega chain indicates the β configuration, R is hydrogen or a saturated or unsaturated acyclic hydrocarbon group having from 1 to about 20 carbon atoms, or $-(CH_2)_mR_1$ wherein m is 0–10, and R_1 is an aliphatic ring having from about 3 to about 7 carbon atoms, or an aryl or heteroaryl ring having from about 4 to about 10 carbon atoms and wherein the heteroatom is selected from the group consisting of N, O and S.

9. The method of claim 8 wherein the noxious action is the elevated intraocular pressure of glaucoma.

10. The method of claim 8 wherein the noxious action is ischemia associated with glaucoma.

11. The method of claim 8 wherein the noxious action is diabetic retinopathy.

12. The method of claim 8 wherein the noxious action is non-glaucomatous ischemia.

13. The method of claim 8 wherein the noxious action is microangiopathic in nature and is a symptom of the disease chosen from the group consisting of polyarteritis nodosa, giant cell angitis, aortitis syndrome and systemic lupus erythematosus.

14. The method of claim 8 wherein oral administration is used to supply the compound to the mammal systemically.

15. The method of claim 8 wherein intrabulbar injection in the eye is used to supply the compound to the mammal.

16. The method of claim 8 wherein parenteral administration is used to supply the compound to the mammal systemically.

17. The method of claim 8 wherein intramuscular injection is used to supply the compound to the mammal systemically.

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